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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jeffrey SCHLOM and Kwong-Yok TSANG

For: **A HUMAN CYTOTOXIC T-LYMPHOCYTE EPITOPE AND ITS AGONIST**

      EPITOPE FROM THE NON-VARIABLE NUMBER OF TANDEM REPEAT

      SEQUENCE OF MUC-1

Box Provisional Patent Application
Commissioner for Patents
Washington, D.C. 20231

COVER SHEET FOR FILING PROVISIONAL APPLICATION
(37 C.F.R. § 1.51(c)(1))

WARNING: "A provisional application must also include the cover sheet required by § 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this section." 37 C.F.R. 1.53(c)(1).

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional

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CERTIFICATION UNDER 37 C.F.R. 1.10*
(Express Mail label number is mandatory.)   (Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on _December 12, 2003____ (date), in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 Mailing Label Number _EV 343 732 672 US_ addressed to: Commissioner for Patents, Washington, D.C. 20231 – BOX PROVISIONAL APPLICATION.

      __Nicholas A. Zachariades_____   _____
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(Cover Sheet for Filing Provisional Application—page 1 of 6)

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: *"A provisional application is not entitled to the right of priority under 35 U.S.C. 119 or 365(a) or § 1.55, or to the benefit of an earlier filing date under 35 U.S.C. 120, 121 or 365(c) or § 1.78 of any other application. No claim for priority under § 1.78(a)(3) may be made in a design application based on a provisional application. No request under § 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of §§ 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. 1.53(c)(3).*

NOTE: *"No information disclosure statement may be filed in a provisional application." 37 C.F.R. § 1.51(d). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 FR 63951, at 63594.*

NOTE: *"No amendment other than to make the provisional application comply with the patent statute and all applicable regulations, may be made to the provisional application after the filing date of the provisional application." 37 C.F.R. § 1.53(c).*

WARNING: *A provisional application may be abandoned by operation of 35 U.S.C. 111(b)(5) on a Saturday, Sunday, or Federal holiday within the District of Columbia, in which case, a nonprovisional application claiming benefit of the provisional application under 35 U.S.C. 119(e) must be filed no later than the preceding day that is not a Saturday, Sunday, or Federal holiday within the District of Columbia. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,202.*

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.51(c)(1)(i).

     1.   The following comprises the information required by 37 C.F.R. § 1.51(c)(1):

     2.   The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(c)(1)(ii)):

NOTE: *"If the correct inventor or inventors are not named on filing, a provisional application without a cover sheet under § 1.51(c)(1), the later submission of a cover sheet under § 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. § 1.48(f)(2).*

NOTE: *"The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in § 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.*

     The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that § 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

See 37 C.F.R. § 1.53.

1.	Jeffrey		SCHLOM
	GIVEN NAME	**MIDDLE INITIAL OR NAME**	**FAMILY (OR LAST) NAME**
2.	Kwong-Yok		TSANG
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3.			
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4.			
	GIVEN NAME	**MIDDLE INITIAL OR NAME**	**FAMILY (OR LAST) NAME**
5.			
	GIVEN NAME	**MIDDLE INITIAL OR NAME**	**FAMILY (OR LAST) NAME**

3.   Residence address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(c)(1)(iii)):

1.   10301 Sorrel Avenue, Potomac, MD 20854
2.   5420 York Lane, Bethesda, MD 20814

4.   The title of the invention is (37 C.F.R. § 1.51(c)(1)(iv)):

METHODS FOR PROPHYLAXIS AND TREATMENT OF HER-2/*neu* TUMORS

5.   The name, registration, customer and telephone numbers of the practitioner *(if applicable)* are (37 C.F.R. § 1.51(c)(1)(v)):

Name of practitioner: Peter F. Corless

Reg. No.   33,860

Tel.:   (617) 439-4444

Customer No.   21874

(complete the following, if applicable)

[ ]   A power of attorney accompanies this cover sheet.

6.   The docket number used to identify this application is (37 C.F.R. § 1.51(c)(1)(vi)):

      Docket No.: 59849-P (47992)

7.   The correspondence address for this application is (37 C.F.R. § 1.51(c)(1)(vii)):
      _Peter F. Corless_____
      _EDWARDS & ANGELL, LLP_____
      _P.O. Box 9169_____
      _Boston, MA 02209_____

8.   Statement as to whether invention was made by an agency of the U.S. Government or under
      contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(c)(1)(viii)).

      This invention was made by an agency of the United States Government, or under contract with an
      agency of the United States Government.

                   [ ]   No
                   [X]   Yes

            The name of the U.S. Government agency and the Government contract number are:
            _____United Sates National Institutes of Health_____
            _____

9.   Identification of documents accompanying this cover sheet:

      A. Documents required by 37 C.F.R. §§ 1.51(c)(2)-(3):

                   Specification: No. of pages __92____
                   Drawings: No. of sheets __4_____

      B.   Additional documents:

                   [X]   Claims: No. of claims __62____
                   [X]   Abstract   No. of sheets _____1____

Note: See 37 C.F.R. § 1.51.

                   [ ]   Power of attorney
                   [ ]   Small entity statement
                   [ ]   Assignment
                   [ ]   Other

10.   Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

    [ ]   Applicant is a small entity.
    [X]   Applicant is not a small entity.

11.   Small entity statement

    [ ]   The statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

12.   Fee payment

    [X]   Fee payment in the amount of $ _160.00_ is being made at this time.

    [ ]   No filing fee is to be paid at this time. (This and the surcharge required by 37 C.F.R. § 1.16(l) can be paid subsequently.)

13.   Method of fee payment

        [X]   Check in the amount of $ _160.00_.
        [ ]   Charge Account No. _____, in the amount of $ _____.
        A duplicate of this Cover Sheet is attached.

Please charge Account No. _04-1105_ for any fee deficiency.

Date: December 12, 2003

Signature of practitioner

 Peter F. Corless
(type or print name of practitioner)

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BOS2_358261.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
NEW NON-PROVISIONAL PATENT APPLICATION

TITLE: A HUMAN CYTOTOXIC T-LYMPHOCYTE EPITOPE AND ITS
AGONIST EPITOPE FROM THE NON-VARIABLE NUMBER OF TANDEM
REPEAT SEQUENCE OF MUC-1

INVENTOR: Jeffrey **SCHLOM** and Kwong-Yok **TSANG**

FILING DATE: December 12, 2003

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A Human Cytotoxic T-Lymphocyte Epitope and its Agonist Epitope from the Non-
Variable Number of Tandem Repeat Sequence of MUC-1

FIELD OF THE INVENTION

10   CTL epitope sequences outside traditional MUC-1 immunogenic tumor antigens
and HLA-anchor residues have been identified. In particular, the invention describes a
method for T-cell activation by modifying HLA-anchor residues to provide a stronger
immune response to native antigens associated with solid tumors, leukemias, or
lymphomas.

15

BACKGROUND OF THE INVENTION

        The tumor-associated antigen MUC-1, or DF-3/MUC-1, is overexpressed on the
cell surface of many human adenocarcinomas such as ovarian, breast, pancreas,
colorectal and prostate carcinoma, and hematological malignancies including multiple
20   myeloma and some B-cell non-Hodgkin lymphomas. While MUC-1 is expressed on
some normal epithelial tissue on lumenal surfaces, it has been demonstrated that the
apical localization of MUC-1 is lost in tumor tissues. In addition, MUC-1 is under-
glycosylated in human adenocarcinomas as compared with normal tissues and thus the
antigenic epitopes of the protein core are more exposed. A high level of MUC-1
25   expression and secretion has also been shown to be associated with poor prognosis and
high metastatic potential. It was initially demonstrated that histocompatibility complex
(MHC)-unrestricted cytotoxic T cells could be established from patients with pancreatic
carcinoma, ovarian cancer and multiple myeloma; these T cells were shown to recognize
the MUC-1 protein core in the 20 amino acid variable number of tandem repeat
30   (VNTR)[2] region. While the VNTR region is immunogenic for MHC non-restricted CTL
as well as for the production of MUC-1 specific antibodies , relatively limited

information is available with respect to the immunogenicity of the region outside the VNTR.

Current treatment of cancers include radiation therapy and chemotherapy, which
5   have particularly adverse effects on a patient undergoing such therapies.

Accordingly, there is a need for improved, safer treatments that have long-lasting protective effects for the prevention and treatment of tumors. In particular, there is a need for treatments that are more specific and less toxic than the currently available
10   therapeutic agents.

SUMMARY OF THE INVENTION
The invention describes the identification and characterization of anti-tumor cytotoxic T lymphocyte (CTL) epitopes. In particular, MUC-1 CTL epitopes in the non-
15   variable number of tandem repeat (VNTR) region extracellular region of MUC-1 are described. The VNTR is not a region of the MUC-1 which is traditionally known to have immunogenic epitopes.. The invention also describes the generation of enhancer agonist epitopes which generate stronger immune cell reaction than native peptides.

20   In a preferred embodiment, the invention provides an isolated nucleic acid molecule which encodes an agonist polypeptide antigen derived from a tumor antigen, such as for example, MUC-1, wherein the agonist polypeptide stimulates a stronger immune response as compared to a native polypeptide.

25   In another preferred embodiment, the agonist polypeptide binds to HLA molecules with a high avidity as compared to native polypeptides. Preferably, the agonist polypeptide has a higher association constant (K_a) for HLA molecules than a native polypeptide. Also preferably, the agonist polypeptide has a lower dissociation constant (K_d) for HLA molecules than a native polypeptide.

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In another preferred embodiment, the nucleic acid molecule encodes an agonist polypeptide up to about 12 amino acids in length. Preferably, the agonist polypeptide is derived from a mucin tumor antigen.

In another preferred embodiment, the agonist polypeptide is derived from a non-variable number of tandem repeats region of MUC-1. Preferably, the agonist polypeptide generates an immune response.

In one aspect of the invention, the generated immune response is a cellular immune response. Cellular immune responses include cytotoxic T cell responses, T helper cell responses, and B cell immune responses.

In another preferred embodiment, the invention provides a nucleic acid molecule comprising a nucleic acid sequence corresponding to (*i.e.* that can code for) any one of the amino acid sequences as identified by SEQ ID NO: 1 through 23, fragments or variants thereof. SEQ ID NO: 1 through 23 are identified by SEQ ID NO: 1 = ATWGQDVTSV; SEQ ID NO: 2 = ALWGQDVTSV; SEQ ID NO: 3 = ALLVLVCVLV; SEQ ID NO: 4 = TISDVSVSDV; SEQ ID NO: 5 = ALAIVYLIAL; SEQ ID NO: 6 = VLVALAIVYL; SEQ ID NO: 7 = YLIALAVCQC; SEQ ID NO: 8 = YLSGADLNL; SEQ ID NO: 9 = WGQDVTSVPV; SEQ ID NO: 10 = REGTINVHDV; SEQ ID NO: 11 = GTQSPFFLLL; SEQ ID NO: 12 = LAFREGTINV; SEQ ID NO: 13 = TLASHSTKTD; SEQ ID NO: 14 = LQRDISEMFL; SEQ ID NO: 15 = ALLVLVCVLV; SEQ ID NO: 16 = TISDVSVSDV; SEQ ID NO: 17 = ALAIVYLIAL; SEQ ID NO: 18 = AIWGQDVTSV; SEQ ID NO: 19 = YLIALAVCQC; SEQ ID NO: 20 = ALWGQDVTSL; SEQ ID NO: 21 = AMWGQDVTSV; SEQ ID NO: 22 = AMWGQDVTSL; SEQ ID NO: 23 = AIWGQDVTSL.

In another preferred embodiment, the invention provides for a vector comprising an isolated nucleic acid molecule expressing any one of amino acids identified by SEQ ID NO: 1 through 23.

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In another preferred embodiment, the vector comprises nucleic acid molecules encoding immune cell co-stimulatory molecules, such as for example, B7-1, ICAM-1 and LFA-331.

5   In yet another preferred embodiment, the invention provides for the transduction of dendritic cells with a vector comprising any one of the molecules as identified by SEQ ID NO: 1 through 23, fragments or variants thereof, and optionally, immune cell co-stimulatory molecules, such as for example, B7-1, ICAM-1 and LFA-3.35.

10   In one aspect of the invention, dendritic cells transduced with the vector comprising any one of the molecules as identified by SEQ ID NO: 1 through 23, fragments or variants thereof, and optionally, immune cell co-stimulatory molecules, generates an immune response, such as activation of a cytotoxic T cell response.

15   In another preferred embodiment, the invention provides a nucleic acid vector comprising one or more nucleic acid sequences encoding polypeptides as identified by any one of SEQ ID NO: 1 through 23, fragments or variants thereof, operably linked to an inducible promoter.

20   In another preferred embodiment the nucleic acid vector is a viral vector, plasmid and the like. Preferably the nucleic acid vector comprises an inducible promoter which is tissue specific, and optionally, immune cell co-stimulatory molecules.

       In another preferred embodiment, the vector comprising a nucleic acid sequence
25   encoding any one of the polypeptides identified by SEQ ID NO: 1 through 23.

       In another preferred embodiment, the vector codes for any one of the polypeptides identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%,
30   even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%.

In another preferred embodiment, the invention provides a host cell expressing the polypeptide products of the vector as identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%. Preferably the host cell is an antigen presenting cell, such as for example, a monocyte/macrophage, dendritic cell or the like.

In another preferred embodiment, the invention provides a method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising administering to a patient any one of the peptides identified by SEQ ID NO: 1 through 23, fragments or variants thereof..

In another preferred embodiment, the invention provides a method for generating an immune response to a MUC-1 tumor antigen comprising administering an isolated nucleic molecule in a therapeutically effective dose sufficient to generate a cellular immune response, wherein the isolated nucleic acid molecule encodes any one of polypeptides identified by SEQ ID NO: 1 through 23., fragments or variants thereof, and optionally immune cell co-stimulatory molecules. Preferably, the vector can express polypeptides as identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%.

In another preferred embodiment, the invention provides for a method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising isolating dendritic cells from a patient suffering from cancer; and, treating the dendritic cells with one or more of the polypeptides identified by SEQ ID NO: 1 through 23; fragments, and variants thereof. Preferably, the treated dendritic cells are administered to the patient.

In another preferred embodiment, the invention provides a method for generating an immune response to a weakly immunogenic antigen comprising

administering to an individual a polypeptide with a high avidity for HLA fused to the weak immunogen.

In one aspect of the invention, the polypeptide comprises the HLA binding fragment of SEQ ID NO: 2.

In another aspect of the invention, the weak immunogen is a differentiation antigen, or a tumor antigen.

In another preferred embodiment, the HLA binding fragment of SEQ ID NO: 2 is fused to a carcinoembryonic antigen, tumor antigen, self antigen, viral antigen and the like.

In another preferred embodiment, the invention provides for an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1 through 23, fragments or variants thereof.

In another preferred embodiment, the invention provides for a polypeptide identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%.

In one aspect of the invention, the polypeptide comprises SEQ ID NO: 2. Preferably, the polypeptide binds to HLA molecules with a high avidity and has a higher association constant (K_a) for the HLA than a native polypeptide and/or a lower dissociation constant (K_d) for the HLA than a native polypeptide.

In another aspect of the invention, the polypeptide is derived from a mucin tumor antigen, preferably, the polypeptide is derived from a non-variable number of tandem repeats region of MUC-1.

In another aspect of the invention, antigen presentation, by antigen presenting cells of the polypeptides induces an immune response, preferably a cellular immune response. For example, the cellular immune response is a cytotoxic T cell response, a T helper cell response, or a B cell immune response.

In another preferred embodiment, the invention provides for an agonist polypeptide comprising an amino acid sequence which is at least about 60% identical to the amino acid sequence of SEQ ID NO: 1 through 23, fragments, or variants thereof, more preferably, the agonist polypeptide comprises an amino acid sequence which is at least about 80% identical to the amino acid sequence of SEQ ID NO: 1 through 23., more preferably, the agonist polypeptide comprises an amino acid sequence which is at least about 90%, 95%, or 99.9% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

Other aspects of the invention are described *infra*.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are graphs showing binding of MUC-1 peptide P-92 and the agonists P-93L and P-93I to HLA-A2 molecule. Figure 1A is a graph showing use of peptides at concentrations of 0-50 μg/ml. Figure 1B is a graph showing use of peptides at concentrations of 0-12.5 μg/ml. P-92 MUC-1 peptide (open square), P-93L (closed square), P-93I (closed triangle). Results are expressed in mean fluorescence intensity (MFI).

Figure 2 is a graph showing the comparison of the stability of the complex of the P-92, P-93L or P-93I peptide with HLA-A2. T2 cells were incubated overnight with P-92 (open square), P-93L (closed square) or P-93I (closed triangle) peptide at a concentration of 50μg/ml and then washed free of unbound peptide and incubated with brefeldin-A to block delivery of new class I molecules to the cell surface. At the indicated times, cells were stained for the presence of surface peptide-HLA-A2

complexes. Results are expressed in relative percentage of binding compared with 100% at time 0.

Figure 3 is a graph showing the ability of autologous B cells pulsed with native MUC-1 peptide P-92 (open square), P-93L peptide (closed square) and P-93I peptide (closed triangle) to induce IFN-γ production by MUC-1–specific T cells. Peptides were used at concentrations of 0-6.25 μg/ml. Results are expressed in pg/ml.

Figure 4 is a graph showing the cytotoxicity of the MUC-1–specific T-cell lines against C1R-A2 cells pulsed with P-92 and P-93L peptide. T-1191-P-93L against C1R-A2 pulsed with P-93L peptide (closed square), T-1191-P-93L against C1R-A2 pulsed with P-92 peptide (open square), T-1191-P-93L against C1R-A2 pulsed with CAP1-6D peptide (closed circle), T-1191-P-92 against C1R-A2 pulsed with P-93L peptide (closed triangle), T-1191-P-92 against C1R-A2 pulsed with P-92 peptide (open triangle), T-1191-P-92 against C1R-A2 pulsed with CAP1-6D peptide (open circle). E: T ratio = 25:1 and 12.5:1 in a 16-h ^{111}In release assay. Bars = SD.

DETAILED DESCRIPTION OF THE INVENTION

We describe herein, *inter alia*, the identification of a novel class I HLA-A2 epitopes of MUC-1 that reside outside of the variable number of tandem repeat (VNTR) region that are important for immune based therapies in the treatment of cancer. We have demonstrated the ability of these epitopes to activate human T cells as measured by IFN-γ production. In particular, one epitope, ATWGQDVTSV (SEQ ID NO: 1), at amino acid position 92-101 and designated P-92), demonstrated the highest level of binding the HLA-A2 and which induced the highest level of IFN-γ in human T cells. The invention also provides for the generation of enhancer agonist epitopes, as identified by epitope, ALWGQDVTSV, (SEQ ID NO: 2; designated P-93L).

The following definitions of certain terms that are used herewith, are set forth as follows.

8

As used herein, "molecule" is used generically to encompass any vector, antibody, protein, drug and the like which are used in therapy and can be detected in a patient by the methods of the invention. For example, multiple different types of nucleic acid delivery vectors encoding different types of genes which may act together to

5   promote a therapeutic effect, or to increase the efficacy or selectivity of gene transfer and/or gene expression in a cell. The nucleic acid delivery vector may be provided as naked nucleic acids or in a delivery vehicle associated with one or more molecules for facilitating entry of a nucleic acid into a cell. Suitable delivery vehicles include, but are not limited to: liposomal formulations, polypeptides; polysaccharides;

10   lipopolysaccharides, viral formulations (e.g., including viruses, viral particles, artificial viral envelopes and the like), cell delivery vehicles, and the like.

As used herein, the term "administering a molecule to a cell" (e.g., an expression vector, nucleic acid, cytokines, a delivery vehicle, agent, and the like) refers to

15   transducing, transfecting, microinjecting, electroporating, or shooting, the cell with the molecule. In some aspects, molecules are introduced into a target cell by contacting the target cell with a delivery cell (e.g., by cell fusion or by lysing the delivery cell when it is in proximity to the target cell).

20   A "genetic modification" refers to any addition, deletion or disruption to a cell's normal nucleotides. Any method which can achieve the genetic modification of APCs are within the spirit and scope of this invention. Art recognized methods include viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction.

25

The terms "nucleic acid molecule" or "polynucleotide" will be used interchangeably throughout the specification, unless otherwise specified. As used herein, "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or

30   deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogues thereof, such as

phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA--DNA, DNA-RNA and RNA--RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any

5 particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the

10 strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates

15 otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to the "antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

As used herein, the term "fragment or segment", as applied to a nucleic acid sequence, gene or polypeptide, will ordinarily be at least about 5 contiguous nucleic acid

20 bases (for nucleic acid sequence or gene) or amino acids (for polypeptides), typically at least about 10 contiguous nucleic acid bases or amino acids, more typically at least about 20 contiguous nucleic acid bases or amino acids, usually at least about 30 contiguous nucleic acid bases or amino acids, preferably at least about 40 contiguous nucleic acid

25 bases or amino acids, more preferably at least about 50 contiguous nucleic acid bases or amino acids, and even more preferably at least about 60 to 80 or more contiguous nucleic acid bases or amino acids in length. "Overlapping fragments" as used herein, refer to contiguous nucleic acid or peptide fragments which begin at the amino terminal end of a nucleic acid or protein and end at the carboxy terminal end of the nucleic acid

30 or protein. Each nucleic acid or peptide fragment has at least about one contiguous nucleic acid or amino acid position in common with the next nucleic acid or peptide

10

fragment, more preferably at least about three contiguous nucleic acid bases or amino acid positions in common, most preferably at least about ten contiguous nucleic acid bases amino acid positions in common.

5   A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 20 nucleotides, more generally at least 23 nucleotides, ordinarily at least 26 nucleotides, more ordinarily at least 29 nucleotides, often at least 32 nucleotides, more often at least 35 nucleotides, typically at least 38 nucleotides, more typically at least 41 nucleotides, usually at least 44 nucleotides, more

10   usually at least 47 nucleotides, preferably at least 50 nucleotides, more preferably at least 53 nucleotides, and in particularly preferred embodiments will be at least 56 or more nucleotides.

A "vector" is a composition which can transduce, transfect, transform or infect a

15   cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a

20   nucleic acid into a cell. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector

25   which encodes a nucleic acid capable of stable replication and expression in a cell once the nucleic acid is transduced into the cell.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, promoters,

30   silencing elements, which induce, inhibit or control transcription of protein coding sequences with which they are operably linked.

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As used herein, the term "downstream" when used in reference to a direction along a nucleotide sequence means in the direction from the 5' to the 3' end. Similarly, the term "upstream" means in the direction from the 3' to the 5' end.

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As used herein, the term "gene" means the gene and all currently known variants thereof and any further variants which may be elucidated.

The term "variant," when used in the context of a polynucleotide sequence, may
10   encompass a polynucleotide sequence related to a wild type gene. This definition may also include, for example, "allelic", "splice," "species," or "polymorphic" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional
15   functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. Of particular utility in the invention are variants of wild type target genes. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene
20   may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

25   As used herein, "variant" of polypeptides refers to an amino acid sequence that is altered by one or more amino acid residues. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan). Analogous
30   minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted

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without abolishing biological activity may be found using computer programs well known in the art, for example, LASERGENE software (DNASTAR).

The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs,) or single base mutations in which the polynucleotide sequence varies by one base.

The terms, "complementary" or "complements" are used interchangeably throughout and mean that two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence. Normally, the complementary sequence of the oligonucleotide has at least 80% or 90%, preferably 95%, most preferably 100%, complementarity to a defined sequence. Preferably, alleles or variants thereof can be identified. A BLAST program also can be employed to assess such sequence identity.

The term "complementary sequence" as it refers to a polynucleotide sequence, relates to the base sequence in another nucleic acid molecule by the base-pairing rules. More particularly, the term or like term refers to the hybridization or base pairing between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 95% of the nucleotides of the other strand, usually at least about 98%, and more preferably from about 99 % to about 100%. Complementary polynucleotide sequences can be identified

by a variety of approaches including use of well-known computer algorithms and software, for example the BLAST program.

A "heterologous" component refers to a component that is introduced into or produced within a different entity from that in which it is naturally located. For example, a polynucleotide derived from one organism and introduced by genetic engineering techniques into a different organism is a heterologous polynucleotide which, if expressed, can encode a heterologous polypeptide. Similarly, a promoter or enhancer that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous promoter or enhancer. Possible alternative terminology includes "foreign" or "exogenous". A heterologous nucleotide sequence may encode a sequence of amino acids, i.e. a peptide or a polypeptide.

A "promoter," as used herein, refers to a polynucleotide sequence that controls transcription of a gene or coding sequence to which it is operably linked. A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources, are well known in the art and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources).

An "enhancer," as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. A large number of enhancers, from a variety of different sources are well known in the art and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences.

"Operably linked" refers to a juxtaposition, wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter is operably linked to a coding sequence if the promoter controls transcription of the coding

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sequence. Although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within or downstream of coding

5   sequences. A polyadenylation sequence is operably linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence.

"Gene delivery," "gene transfer," and the like as used herein, are terms referring

10 to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgenes") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked"

15 polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an

20 extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

As used herein, a "target cell" or "recipient cell" refers to an individual cell or

25 cell which is desired to be, or has been, a recipient of exogenous nucleic acid molecules, polynucleotides and/or proteins. The term is also intended to include progeny of a single cell.

"*In vivo*" gene delivery, gene transfer, gene therapy and the like as used herein,

30 are terms referring to the introduction of a vector comprising an exogenous polynucleotide directly into the body of an organism, such as a human or non-human

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mammal, whereby the exogenous polynucleotide is introduced to a cell of such organism *in vivo.*

A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into
5   the cell from the extracellular environment. Any method of transferring a nucleic acid
into the cell may be used; the term, unless otherwise indicated, does not imply any
particular method of delivering a nucleic acid into a cell. A cell is "transformed" by a
nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A
vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. A
10 vector optionally includes materials to aid in achieving entry of the nucleic acid into the
cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction
vector" is a vector which encodes a nucleic acid capable of stable replication and
expression in a cell once the nucleic acid is transduced into the cell.

15 As used herein, "homologous recombination" means a nucleotide sequence on
one vector is homologous to a nucleotide sequence on another vector. Using restriction
enzymes to cut the two sequences and ligating the two sequences results in the two
vectors combining. Typically, several kilobases of unaltered flanking DNA (both at the
5' and 3' ends) are included in the vector (see, *e.g.*, Thomas and Capecchi, *Cell*, 51:503
20 (1987) for a description of homologous recombination vectors).

Homologous nucleic acid sequences, when compared, exhibit significant
sequence identity or similarity. The standards for homology in nucleic acids are either
measures for homology generally used in the art by sequence comparison or based upon
25 hybridization conditions. The hybridization conditions are described in greater detail
below.

"Stringency" is meant the combination of conditions to which nucleic acids are
subject that cause the duplex to dissociate, such as temperature, ionic strength, and
30 concentration of additives such as formamide. Conditions that are more likely to cause

the duplex to dissociate are called "higher stringency", e.g. higher temperature, lower ionic strength and higher concentration of formamide.

For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50° C. to about 70° C.

For certain applications, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37° C. to about 55° C., while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20° C. to about 55° C. Thus, hybridization conditions can be readily manipulated depending on the desired results.

The phrase "hybridizing conditions" and its grammatical equivalents, when used with a maintenance time period, indicates subjecting the hybridization reaction admixture, in context of the concentration of the reactants and accompanying reagents in the admixture, to time, temperature, pH conditions sufficient to allow the polynucleotide probe to anneal with the target sequence, typically to form the nucleic acid duplex. Such time, temperature and pH conditions required to accomplish the hybridization depend, as is well known in the art on the length of the polynucleotide probe to be hybridized, the degree of complementarity between the polynucleotide probe and the target, the guanidine and cytosine content of the polynucleotide, the stringency of the hybridization desired, and the presence of salts or additional reagents in the hybridization reaction admixture as may affect the kinetics of hybridization. Methods for optimizing hybridization conditions for a given hybridization reaction admixture are well known in the art.

As used herein, "substantial homology" in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least 56%, more generally at least 59%, ordinarily at least 62%, more ordinarily at least 65%, often at least 68%, more often at least 71%, typically at least 74%, more typically at least 77%, usually at least 80%, more usually at least about 85%, preferably at least about 90%, more preferably at least about 95 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a fragment derived from SEQ ID NO: 1. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See Kanehisa (1984) *Nuc. Acids Res.* 12:203-213. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides. The endpoints of the segments may be at many different pair combinations.

The term "polymorphism" refers to the coexistence of more than one form of a gene or portion (e.g., allelic variant) thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A specific genetic sequence at a polymorphic region of a gene is an allele. A polymorphic region can be a single nucleotide, the identity of which differs in different alleles. A polymorphic region can also be several nucleotides long.

As used herein, the term "specifically hybridizes" or "specifically detects" refers to the ability of a nucleic acid molecule to hybridize to at least approximately 6 consecutive nucleotides of a sample nucleic acid.

5   "Substantially purified" refers to nucleic acid molecules or proteins that are removed from their natural environment and are isolated or separated, and are at least about 60% free, preferably about 75% free, and most preferably about 90% free, from other components with which they are naturally associated.

10   An "antigen" is any substance that reacts specifically with antibodies or T lymphocytes (T cells). An "antigen-binding site" is the part of an immunoglobulin molecule that specifically binds an antigen. Additionally, an antigen-binding site includes any such site on any antigen-binding molecule, including, but not limited to, an MHC molecule or T cell receptor. "Antigen processing" refers to the degradation of an

15   antigen into fragments (*e.g.*, the degradation of a protein into peptides) and the association of one or more of these fragments (*e.g.*, via binding) with MHC molecules for presentation by "antigen-presenting cells" to specific T cells.

      "Dendritic cells" (DC) are potent antigen-presenting cells, capable of triggering a

20   robust adaptive immune response *in vivo*. It has been shown that activated, mature DC provide the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex

25   ("MHC" defined above) class I or II protein on the surface of APCs. The second type of signal, called a co-stimulatory signal, is neither antigen-specific nor MHC- restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals. This two-fold signaling can, therefore, result in a vigorous immune response. As noted *supra*, in most non-avian

30   vertebrates, DC arise from bone marrow-derived precursors. Immature DC are found in the peripheral blood and cord blood and in the thymus. Additional immature

populations may be present elsewhere. DC of various stages of maturity are also found in the spleen, lymph nodes, tonsils, and human intestine. Avian DC may also be found in the bursa of Fabricius, a primary immune organ unique to avians. In a preferred embodiment, the dendritic cells of the present invention are mammalian, preferably human, mouse, or rat.

A "co-stimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a peptide/MHC complex bound by a T cell receptor on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide.

As used herein, "immunoreceptors" will refer to class I MHC (HLA-A, -B, -C, - G) and the like) and other immune related receptors, such as for example Gp49, PIR, PIRA, PIRB, LIR, NKR-P1, NKp46, Digr1, ILT, MIR, KIR and the like. MHC may also include other classes such as MHC class II and MHC class III, derivatives and mutants thereof. The human MHC complex is also called the human leukocyte antigen (HLA) complex. MHC antigens are divided into MHC class I antigens (in humans, this class includes HLA-A, -B, and -C antigens) and MHC class II antigens (in humans, this class includes HLA-DP, -DQ, and -DR antigens). Thus, the terms "MHC-II antigens", "MHC class II antigens", and "MHC class II transplantation antigens" are used interchangeably herein to refer to the class of proteins, which in humans, includes HLA-DP, -DQ and -DR antigens. While the terms "MHC class II genes" and "MHC-II genes" are used interchangeably herein to refer to the genes which encode the MHC class II transplantation antigens. The term "MHC-II" is used herein to refer to the gene locus which encodes the MHC class II transplantation antigens, as well as the group of proteins encoded by that locus. Transplantation antigens also include cell surface molecules other than MHC class I and II antigens. These antigens include the following: (1) the ABO antigens involved in blood cell recognition; (2) cell adhesion molecules such as ICAM, which is involved in leukocyte cell-cell recognition; and (3) $\beta2$ - microglobulin, a polypeptide associated with the 44 kd heavy chain polypeptide that comprises the HLA-I antigens but is not encoded by the MHC complex. HLA

haplotypes/allotypes vary from individual to individual and it is often helpful to
determine the individual's HLA type. The HLA type may be determined via standard
typing procedures and the peripheral blood lymphocytes (PBLs) purified by Ficoll
gradients.

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"Diagnostic" or "diagnosed" means identifying the presence or nature of a
pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The
"sensitivity" of a diagnostic assay is the percentage of diseased individuals who test
positive (percent of "true positives"). Diseased individuals not detected by the assay are
10   "false negatives." Subjects who are not diseased and who test negative in the assay, are
termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false
positive rate, where the "false positive" rate is defined as the proportion of those without
the disease who test positive. While a particular diagnostic method may not provide a
definitive diagnosis of a condition, it suffices if the method provides a positive
15   indication that aids in diagnosis.

The terms "patient" or "individual" are used interchangeably herein, and is meant
a mammalian subject to be treated, with human patients being preferred. In some cases,
the methods of the invention find use in experimental animals, in veterinary application,
20   and in the development of animal models for disease, including, but not limited to,
rodents including mice, rats, and hamsters; and primates.

"Label molecules" are chemical or biochemical moieties used for labeling a
polynucleotide, a polypeptide, or an antibody. They include, but are not limited to,
25   radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents,
chromogenic agents, chemiluminescent agents, magnetic particles, and the like. Reporter
molecules specifically bind, establish the presence of, and allow quantification of a
particular polynucleotide, polypeptide, or antibody.

30   "Sample" is used herein in its broadest sense. A sample comprising
polynucleotides, polypeptides, peptides, antibodies and the like may comprise a bodily

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fluid; a soluble fraction of a cell preparation, or media in which cells were grown; a chromosome, an organelle, or membrane isolated or extracted from a cell; genomic DNA, RNA, or cDNA, polypeptides, or peptides in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, skin or hair; and the like.

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As used herein, "fresh tumors" refer to tumors removed from a host by surgical or other means.

As used herein, "proliferative growth disorder, "neoplastic disease," "tumor",
10   "cancer" are used interchangeably as used herein refers to a condition characterized by uncontrolled, abnormal growth of cells. Preferably the cancer to be treated is MUC-1 positive cancer and the abnormal proliferation of cells can be any cell in the organ. Examples of cancer include but are not limited to, carcinoma, blastoma, and sarcoma. As used herein, the term "carcinoma" refers to a new growth that arises from epithelium,
15   found in skin or, more commonly, the lining of body organs.

"Treatment" is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative
20   measures. "Treatment" may also be specified as palliative care. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy.

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The term "in need of such treatment" as used herein refers to a judgment made by a care giver such as a physician, nurse, or nurse practitioner in the case of humans that a patient requires or would benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a care giver's expertise, but that include the
30   knowledge that the patient is ill, or will be ill, as the result of a condition that is treatable by the compositions of the invention.

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"Cells of the immune system" or "immune cells" as used herein, is meant to include any cells of the immune system that may be assayed, including, but not limited to, B lymphocytes, also called B cells, T lymphocytes, also called T cells, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages, neutrophils, granulocytes, mast cells, platelets, Langerhans cells, stem cells, dendritic cells, peripheral blood mononuclear cells, tumor-infiltrating (TIL) cells, gene modified immune cells including hybridomas, drug modified immune cells, and derivatives, precursors or progenitors of the above cell types.

"Immune effector cells" refers to cells capable of binding an antigen and which mediate an immune response. These cells include, but are not limited to, T cells (T lymphocytes), B cells (B lymphocytes), monocytes, macrophages, natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates.

"T cells" or "T lymphocytes" are a subset of lymphocytes originating in the thymus and having heterodimeric receptors associated with proteins of the CD3 complex (e.g., a rearranged T cell receptor, the heterodimeric protein on the T cell surfaces responsible for antigen/MHC specificity of the cells). T cell responses may be detected by assays for their effects on other cells (e.g., target cell killing, macrophage, activation, B-cell activation) or for the cytokines they produce.

The term "activated T cell," as used herein, refers to a T cell that expresses antigens indicative of T-cell activation (that is, T cell activation markers). Examples of T cell activation markers include, but are not limited to, CD25, CD26, CD30, CD38, CD69, CD70, CD71, ICOS, OX-40 and 4-1BB. The expression of activation markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

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The term "resting T cell," as used herein, refers to a T cell that does not express T-cell activation markers. Resting T cells include, but are not limited to, T cells which are CD25⁻, CD69⁻, ICOS⁻, SLAM⁻, and 4-1BB⁻. The expression of these markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

"CD4" is a cell surface protein important for recognition by the T cell receptor of antigenic peptides bound to MHC class II molecules on the surface of an APC. Upon activation, naïve CD4 T cells differentiate into one of at least two cell types, Th1 cells and Th2 cells, each type being characterized by the cytokines it produces. "Th1 cells" are primarily involved in activating macrophages with respect to cellular immunity and the inflammatory response, whereas "Th2 cells" or "helper T cells" are primarily involved in stimulating B cells to produce antibodies (humoral immunity). CD4 is the receptor for the human immunodeficiency virus (HIV). Effector molecules for Th1 cells include, but are not limited to, IFN-γ, GM-CSF, TNF-α, CD40 ligand, Fas ligand, IL-3, TNF-β, and IL-2. Effector molecules for Th2 cells include, but are not limited to, IL-4, IL-5, CD40 ligand, IL-3, GS-CSF, IL-10, TGF-β, and eotaxin. Activation of the Th1 type cytokine response can suppress the Th2 type cytokine response.

"CD8" is a cell surface protein important for recognition by the T cell receptor of antigenic peptides bound to MHC class I molecules. CD8 T cells usually become "cytotoxic T cells" or "killer T cells" and activate macrophages. Effector molecules include, but are not limited to, perforin, granzymes, Fas ligand, IFN-γ, TNF-α, and TNF-β.

The terms "specific binding" or "specifically binding", as used herein, in reference to the interaction of an antibody and a protein or peptide, mean that the interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the protein; in other words, the antibody is recognizing and

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binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A", the presence of a protein comprising epitope A (or free, unlabeled A) in a reaction comprising labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody. "Specific binding" in general, refers to any immune related molecule binding to its ligand, such as for example the binding of a T cell receptor expressed by a T lymphocyte, to an MHC molecule and peptide on an antigen presenting cell.

"Activity", "activation" or "augmentation" is the ability of immune cells to respond and exhibit, on a measurable level, an immune function. Measuring the degree of activation refers to a quantitative assessment of the capacity of immune cells to express enhanced activity when further stimulated as a result of prior activation. The enhanced capacity may result from biochemical changes occurring during the activation process that allow the immune cells to be stimulated to activity in response to low doses of stimulants.

Immune cell activity that may be measured include, but is not limited to, (1) cell proliferation by measuring the cell or DNA replication; (2) enhanced cytokine production, including specific measurements for cytokines, such as IFN-γ, GM-CSF, or TNF-α; (3) cell mediated target killing or lysis; (4) cell differentiation; (5) immunoglobulin production; (6) phenotypic changes; (7) production of chemotactic factors or chemotaxis, meaning the ability to respond to a chemotactin with chemotaxis; (8) immunosuppression, by inhibition of the activity of some other immune cell type; and, (9) apoptosis, which refers to fragmentation of activated immune cells under certain circumstances, as an indication of abnormal activation.

An "adjuvant" is any substance capable of enhancing the immune response to an antigen with which it is mixed. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, KLH, and

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dinitrophenol, as well as BCG (bacilli Calmette-Guerin) and *Corynabacterium parvum*, which are often used in humans, and ligands of CCR6 and other chemokine receptors.

A "chemokine" is a small cytokine involved in the migration and activation of cells, including phagocytes and lymphocytes, and plays a role in inflammatory responses. Three classes of chemokines have been defined by the arrangement of the conserved cysteine (C) residues of the mature proteins: the CXC or α chemokines that have one amino acid residue separating the first two conserved cysteine residues; the CC or β chemokines in which the first two conserved cysteine residues are adjacent; the C or γ chemokines which lack two (the first and third) of the four conserved cysteine residues. Within the CXC subfamily, the chemokines can be further divided into two groups. One group of the CXC chemokines have the characteristic three amino acid sequence ELR (glutamic acid-leucine-arginine) motif immediately preceding the first cysteine residue near the amino terminus. A second group of CXC chemokines lack such an ELR domain. The CXC chemokines with the ELR domain (including IL-8, GROα/β/γ, mouse KC, mouse MIP-2, ENA-78, GCP-2, PBP/CTAPIII/ β -TG/NAP-2) act primarily on neutrophils as chemoattractants and activators, inducing neutrophil degranulation with release of myeloperoxidase and other enzymes. The CXC chemokines without the ELR domain (e.g., IP-10/mouse CRG, Mig, PBSF/SDF-1, PF4), the CC chemokines (e.g., MIP-1α, MIP-1 β, RANTES, MCP-1/2/3/4/mouse JE/mouse MARC, eotaxin, I-309/TCA3, HCC-1, C10), and the C chemokines (e.g., lymphotactin), chemoattract and activate monocytes, dendritic cells, T-lymphocytes, natural killer cells, B-lymphocytes, basophils, and eosinophils.

A "cytokine" is a protein made by a cell that affect the behavior of other cells through a "cytokine receptor" on the surface of the cells the cytokine effects. Cytokines manufactured by lymphocytes are sometimes termed "lymphokines." Examples of cytokines include interleukins, interferons and the like.

By "immunologically effective" is meant an amount of the peptide or fragment thereof which is effective to activate an immune response to prevent or treat proliferative

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cell growth disorders, such as cancer. Obviously, such amounts will vary between species and individuals depending on many factors. For example, higher doses will generally be required for an effective immune response in a human compared with a mouse.

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As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins containing the sequences recited herein. A polypeptide comprising an epitope of a protein containing a sequence as described herein may consist entirely of the epitope, or may contain additional sequences. The additional sequences may be derived from the native protein or may be heterologous, and such sequences may (but need not) possess immunogenic or antigenic properties.

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one binding domain, where an antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, which allows an immunological reaction with the antigen. Antibodies include recombinant proteins comprising the binding domains, as wells as fragments, including Fab, Fab', F(ab)$_2$, and F(ab')$_2$ fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. It also includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, or single chain antibodies. "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH$_1$, CH$_2$ and CH$_3$, but does not include the heavy chain variable region.

An "epitope", as used herein, is a portion of a polypeptide that is recognized (i.e., specifically bound) by a B-cell and/or T-cell surface antigen receptor. Epitopes may generally be identified using well known techniques, such as those summarized in Paul,

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Fundamental Immunology, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides derived from the native polypeptide for the ability to react with antigen-specific antisera and/or T-cell lines or clones. An epitope of a polypeptide is a portion that reacts with such antisera and/or T-

5 cells at a level that is similar to the reactivity of the full length polypeptide (e.g., in an ELISA and/or T-cell reactivity assay). Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. B-cell and T-cell epitopes may also be predicted via computer analysis.

10 Polypeptides comprising an epitope of a polypeptide that is preferentially expressed in a tumor tissue (with or without additional amino acid sequence) are within the scope of the present invention.

As used herein, the term " agonist polypeptide" refers to epitopes in the

15 polypeptide which activate a stronger immune response than a native polypeptide. Examples of differences in properties between an agonist polypeptide versus a native polypeptide include, but not limited to a) binding HLA molecules at lower peptide concentrations, (b) demonstrate a higher avidity for HLA molecules in dissociation assays, (c) when used with antigen-presenting cells induce the production of more IFN-γ

20 by T cells derived with the use of the native peptide. Increased or augmented immune response are measured as described above.

As used herein, "native polypeptide" refers to a polypeptide as found in its natural environment. For example, a native MUC-1 tumor antigen is expressed by a

25 tumor cell in a patient.

In a preferred embodiment, agonist polypeptides generate stronger immune responses, as compared to the native polypeptide. For example, compared with the native P-92 peptide, agonist polypeptides (a) bind HLA-A2 at lower peptide

30 concentrations, (b) demonstrate a higher avidity for HLA-A2 in dissociation assays, (c) when used with antigen-presenting cells induce the production of more IFN-γ by T cells

derived with the use of the native peptide, and (d) were capable of more efficiently generating MUC-1–specific human T-cell lines from normal volunteers and pancreatic cancer patients. Most importantly, the T-cell lines generated using the agonist epitope were more efficient than those generated with the native epitope, in the lysis of targets pulsed with the native epitope and in the lysis of HLA-A2 human tumor cells expressing MUC-1.

In another preferred embodiment, patients, suffering from or susceptible to tumors, infectious diseases and the like are treated with autologous antigen presenting cells, such as for example dendritic cells (DCs), that have been transduced with a viral vector encoding anyone of the polypeptides as identified by SEQ ID NO: 1 through 23, fragments or variants thereof, optionally expressing co-stimulatory molecules. For example, autologous DCs infected with rF-MUC-1/TRICOM were used as APC. rF-MUC-1/TRICOM is a replication-defective avipox vector containing the transgenes for MUC-1 and for a triad of human costimulatory molecules (B7-1, ICAM-1 and LFA-3, designated TRICOM). rF-MUC-1/TRICOM was shown to efficiently infect human DCs and hyperexpress each of the costimulatory molecules, as well as MUC-1, on the DC surface (Table 2).

In another preferred embodiment, the invention provides a method for generating an immune response to a weakly immunogenic antigen comprising administering to an individual an agonist polypeptide, as identified by any one of SEQ ID NO: 1 through 23, variants or fragments thereof, with a high avidity for HLA fused to the weak immunogen.

In a preferred embodiment, the invention provides an isolated nucleic acid molecule which encodes an agonist polypeptide antigen derived from a tumor antigen, such as for example, MUC-1, wherein the agonist polypeptide stimulates a stronger immune response as compared to a native polypeptide. Other examples of tumor antigens, include, but are not limited to HER2/*neu*, carcinoembryonic antigen (CEA), p53.

In another preferred embodiment, the invention provides a nucleic acid molecule comprising a nucleic acid sequence corresponding to any one of the amino acid sequences as identified by SEQ ID NO: 1 through 23, fragments or variants thereof.

5 SEQ ID NO: 1 through 23 are identified by SEQ ID NO: 1 = ATWGQDVTSV; SEQ ID NO: 2 = ALWGQDVTSV; SEQ ID NO: 3 = ALLVLVCVLV; SEQ ID NO: 4 = TISDVSVSDV; SEQ ID NO: 5 = ALAIVYLIAL; SEQ ID NO: 6 = VLVALAIVYL; SEQ ID NO: 7 = YLIALAVCQC; SEQ ID NO: 8 = YLSGADLNL; SEQ ID NO: 9 = WGQDVTSVPV; SEQ ID NO: 10 = REGTINVHDV; SEQ ID NO: 11 =

10 GTQSPFFLLL; SEQ ID NO: 12 = LAFREGTINV; SEQ ID NO: 13 = TLASHSTKTD; SEQ ID NO: 14 = LQRDISEMFL; SEQ ID NO: 15 = ALLVLVCVLV; SEQ ID NO: 16 = TISDVSVSDV; SEQ ID NO: 17 = ALAIVYLIAL; SEQ ID NO: 18 = AIWGQDVTSV; SEQ ID NO: 19 = YLIALAVCQC; SEQ ID NO: 20 = ALWGQDVTSL; SEQ ID NO: 21 = AMWGQDVTSV; SEQ ID NO: 22 =

15 AMWGQDVTSL; SEQ ID NO: 23 = AIWGQDVTSL.

In another preferred embodiment, the invention provides for a vector comprising an isolated nucleic acid molecule expressing any one of amino acids identified by SEQ ID NO: 1 through 23, fragments or variants, thereof. The vector preferably encodes

20 encoding immune cell co-stimulatory molecules, such as for example, B7-1, ICAM-1 and LFA-331.

In yet another preferred embodiment, the invention provides for the transduction of dendritic cells with a vector comprising any one of the molecules as identified by

25 SEQ ID NO: 1 through 23, fragments or variants thereof, and optionally, immune cell co-stimulatory molecules, such as for example, B7-1, ICAM-1 and LFA-3.35. These recombinant vectors provide specific anti-tumor effect for patients who have been diagnosed with MUC-1$^+$ tumors. However, this antigen is merely an illustrative example and is not meant to be construed as limiting in any way. Examples of other

30 antigens that are useful for treating different types of cancers, include, but not limited to overexpressed or mutated forms of antigens. For example, Her2/*neu*$^+$ tumors such as

breast, renal, prostate, and other HER2 tumors, carcinogenic embryonic antigen (CEA) for gastro-intestinal cancers; K-ras for lung, gastrointestinal and bladder cancers; p53 which affects a wide variety of neoplastic growth; SARDT3 in neck and head cancers.

5   In another preferred embodiment, dendritic cells of an individual, suffering from or susceptible to, cancer, are transduced *in vivo* with recombinant vectors expressing agonist polypeptide epitopes. Dendritic cells can be isolated from a patient, cultured *ex vivo* with a vector, and then re-infusing the cultured dendritic cells into the patient. Culturing of dendritic cells is described in detail in the examples which follow.
10   Alternatively, the vector may be administered to a patient in need of such treatment.

          In a preferred embodiment, transduced dendritic cells present antigen, for example, agonist peptide fragments of the MUC-1 antigen on their surface. Lymphocytes, specific for the presented antigens, are activated, proliferate and recognize
15   tumor cells expressing the MUC-1 antigen. Lymphocytes include, B cells, T helper cells and cytotoxic T cells. Recognition, of any cell expressing antigenic epitopes by the immune cells, results in the destruction of a tumor cell.

          In another preferred embodiment, the invention provides a nucleic acid vector
20   comprising one or more nucleic acid sequences encoding polypeptides as identified by any one of SEQ ID NO: 1 through 23, fragments or variants thereof, operably linked to an inducible promoter.

          In another preferred embodiment the nucleic acid vector is a viral vector, plasmid
25   and the like. Preferably the nucleic acid vector comprises an inducible promoter which is tissue specific, and optionally, immune cell co-stimulatory molecules.

          In another preferred embodiment, the vector comprising a nucleic acid sequence encoding any one of the polypeptides identified by SEQ ID NO: 1 through 23.
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In another preferred embodiment, the vector codes for any one of the polypeptides identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%.

5

In another preferred embodiment, the invention provides a host cell expressing the polypeptide products of the vector as identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or

10   99.9%. Preferably the host cell is an antigen presenting cell, such as for example, a monocyte/macrophage, dendritic cell or the like.

In accordance with the invention, the transduced dendritic cells present antigen to cells of the immune system and activate the immune system to recognize tumor antigen

15   epitopes, such as for example a tumor cell expressing the MUC-1 antigen.

In a preferred embodiment, the vector is a avipox vector comprising nucleic acid molecules encoding agonist polypeptides and co-stimulatory molecules, as described in detail in the examples which follow. Other vectors may also be used. Preferred vectors

20   include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. Viral vectors can be chosen to introduce the genes to cells of choice. Such vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as herpes simplex I virus (HSV) vector (Geller et al., 1995, *J. Neurochem*. 64: 487; Lim et al., 1995, *in*

25   DNA Cloning: Mammalian Systems, D. Glover, ed., Oxford Univ. Press, Oxford, England; Geller et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 1149), other adenovirus vectors (LeGal LaSalle et al., 1993, *Science* 259: 988; Davidson et al., 1993, *Nat. Genet.* 3: 219; Yang et al., 1995, *J. Virol*. 69: 2004) and adeno-associated virus vectors (Kaplitt et al., 1994, *Nat. Genet*. 8: 148; Kotin, et al. WO 98/11244 (3/19/1998) and

30   Chiorini, et al WO 99/61601 (12/2/1999)).

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter

5   term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The vectors can be introduced by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include for example, naked DNA calcium phosphate precipitation, DEAE dextran, electroporation, protoplast fusion, lipofection, cell

10   microinjection and viral vectors.

The vector can be employed to target essentially any desired target cell. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Other methods that can be used include catheters, intravenous,

15   parenteral, intraperitoneal, and subcutaneous injection, and oral or other known routes of administration.

Another preferred method is DNA immunization. DNA immunization employs the subcutaneous injection of a plasmid DNA (pDNA) vector encoding a tumor marker.

20   The pDNA sequence is taken up by antigen presenting cells (APC), preferably by dendritic cells. Once inside the cell, the DNA encoding protein is transcribed and translated and presented to lymphocytes.

Genetic constructs comprise a nucleotide sequence that encodes the nucleic acid

25   sequence of choice and preferably includes an intracellular trafficking sequence operably linked to regulatory elements needed for gene expression.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's

30   chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which

can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells. Gene constructs may be part of genomes of recombinant viral vaccines where the genetic material either integrates into the chromosome of the cell or remains extrachromosomal.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers may be required for gene expression of the sequence of choice, for example, the agonist polypeptides identified by SEQ ID NO: 1 through 23, variants or fragments thereof. It is necessary that these elements be operably linked to the sequence that encodes the desired proteins and that the regulatory elements are operable in the individual to whom they are administered.

Initiation codons and stop codons are generally considered to be part of a nucleotide sequence that encodes the immunogenic target protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter,

Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metallothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. For example, plasmids pCEP4 and pREP4 from Invitrogen (San Diego, Calif.) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

The method of the present invention comprises the steps of administering nucleic acid molecules to tissue of the individual. In some preferred embodiments, the nucleic

acid molecules are administered intramuscularly, intranasally, intraperitoneally, subcutaneously, intradermally, or topically or by lavage to mucosal tissue selected from the group consisting of vaginal, rectal, urethral, buccal and sublingual.

5   In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a facilitating agent. Facilitating agents are also referred to as polynucleotide function enhancers or genetic vaccine facilitator agents. Facilitating agents are described in e.g. International Application No. PCT/US94/00899 filed Jan. 26, 1994 and International Application No. PCT/US95/04071 filed Mar. 30,
10   1995, both incorporated herein by reference. Facilitating agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules.

15   In some preferred embodiments, the genetic constructs of the invention are formulated with or administered in conjunction with a facilitator selected from the group consisting of, for example, benzoic acid esters, anilides, amidines, urethans and the hydrochloride salts thereof such as those of the family of local anesthetics. The facilitating agent is administered prior to, simultaneously with or subsequent to the
20   genetic construct. The facilitating agent and the genetic construct may be formulated in the same composition.

       In some embodiments of the invention, the individual is first subject to injection of the facilitator prior to administration of the genetic construct. That is, for example, up to a about a week to ten days prior to administration of the genetic construct, the
25   individual is first injected with the facilitator. In some embodiments, the individual is injected with the facilitator about 1 to 5 days; in some embodiments 24 hours, before or after administration of the genetic construct. Alternatively, if used at all, the facilitator is administered simultaneously, minutes before or after administration of the genetic
30   construct. Accordingly, the facilitator and the genetic construct may be combined to form a single pharmaceutical composition.

In some embodiments, the genetic constructs are administered free of facilitating agents, that is in formulations free from facilitating agents using administration protocols in which the genetic constructions are not administered in conjunction with the administration of facilitating agents.

Nucleic acid molecules which are delivered to cells according to the invention may serve as genetic templates for proteins that function as prophylactic and/or therapeutic immunizing agents. In preferred embodiments, the nucleic acid molecules comprise the necessary regulatory sequences for transcription and translation of the coding region in the cells of the animal.

In further embodiments of the present invention, the agonist polypeptides described herein may be used for the immunotherapy of MUC-1 positive tumors. In these embodiments, the compounds (which may be polypeptides, antibodies or nucleic acid molecules) are preferably incorporated into pharmaceutical compositions or vaccines. Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier. Vaccines may comprise one or more polypeptides and an immune response enhancer, such as an adjuvant or a liposome (into which the compound is incorporated). Pharmaceutical compositions and vaccines may additionally contain a delivery system, such as biodegradable microspheres which are disclosed, for example, in U.S. Pat. Nos. 4,897,268 and 5,075,109. Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, including one or more separate polypeptides.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose,

glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention.

5   Any of a variety of adjuvants may be employed in the vaccines of this invention to nonspecifically enhance the immune response. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially

10   available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.), Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.), alum, biodegradable microspheres, monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

15   In another preferred embodiment, the invention provides a method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising administering to a patient any one of the peptides identified by SEQ ID NO: 1 through 23, fragments or variants thereof..

20   In accordance with the invention, an immune response to a MUC-1 tumor antigen, is generated, by administering an agonist polypeptides in a therapeutically effective dose sufficient to generate a cellular immune response, wherein the agonist polypeptides are any one of polypeptides identified by SEQ ID NO: 1 through 23., fragments or variants thereof, and optionally immune cell co-stimulatory molecules.

25   Preferably, the polypeptides as identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%.

30   The peptides are administered to an individual suffering from or susceptible to cancers. Definite clinical diagnosis of a particular cancer warrants the administration of

the peptides, including the early stages of the disease. Prophylactic applications are warranted in cases where individuals with familial history of disease and predicted to be at risk by reliable prognostic indicators could be treated prophylactically to interdict cancer prior to onset, such as MUC-1 positive cancer; or can be administered post operatively.

Peptide vaccines can be administered in many possible formulations, in pharmacologically acceptable mediums. In the case of a short peptide, the peptide can be conjugated to a carrier, such as KLH, in order to increase its immunogenicity. The vaccine can be administered in conjunction with an adjuvant, various of which are known to those skilled in the art. After initial immunization with the vaccine, a booster can be provided. The vaccines are administered by conventional methods, in dosages which are sufficient to elicit an immunological response, which can be easily determined by those skilled in the art.

Efficacy of the peptide in the context of prevention is judged based on the following criteria: frequency of peptide reactive T cells determined by limiting dilution, proliferation response of peptide reactive T cell lines and clones, cytokine profiles of T cell lines and clones to the desired peptide established from patients. Efficacy is established by decrease in frequency of reactive cells, a reduction in thymidine incorporation with altered peptide compared to native, and a reduction in TNF and IFN-α. Clinical measurements include the relapse rate in one and two year intervals, on a Kaplan-Meier curve, a delay in sustained cancer stage progression reduction in the number and size of tumors including a change in area and volume of T2 images on MRI, and the number and volume of lesions determined by gadolinium enhanced images.

Peptides, variants and fragments thereof, of the present invention may be administered either alone, or as a pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the peptides, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered

saline, phosphate buffered saline and the like, carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, cytokines like β-interferon.

Compositions of the present invention may be formulated for the manner of administration indicated, including for example, for oral, nasal, venous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration. Within other embodiments of the invention, the compositions described herein may be administered as part of a sustained release implant. Within yet other embodiments, compositions of the present invention may be formulized as a lyophilizate, utilizing appropriate excipients which provide stability as a lyophilizate, and subsequent to rehydration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease. Within particularly preferred embodiments of the invention, the peptides, variants, or fragments thereof, or pharmaceutical compositions described herein may be administered at a dosage ranging from about 5 to 50 mg/kg, although appropriate dosages may be determined by clinical trials. Dosages of peptide analogue will be approximately 5-50 mg/kg, but are determined more accurately following trials. Patients may be monitored for therapeutic effectiveness by MRI, and signs of clinical exacerbation, as described above.

MRI can be used to measure active lesions using gadolinium-DTPA-enhanced imaging (McDonald et al. *Ann. Neurol.* 36:14, 1994) or the location and extent of lesions using T_2 -weighted techniques. Briefly, baseline MRIs are obtained. The same imaging plane and patient position are used for each subsequent study. Positioning and imaging sequences are chosen to maximize lesion detection and facilitate lesion tracing. The

same positioning and imaging sequences are used on subsequent studies. The presence, location and extent of MS lesions are determined by radiologists. Areas of lesions are outlined and summed slice by slice for total lesion area. Three analyses may be done: evidence of new lesions, rate of appearance of active lesions, percentage change in lesion area (Paty et al., *Neurology* 43:665, 1993). Improvement due to therapy is established when there is a statistically significant improvement in an individual patient compared to baseline or in a treated group versus a placebo group.

In another aspect of the invention, any tumor antigen polypeptide can be administered to an individual diagnosed as suffering from or susceptible to cancers. The polypeptides corresponding to identified tumor antigens can be used to stimulate the cells of the immune system to recognize and lyse tumor cells expressing tumor antigens, such as for example, CEA, p53, K-ras, and the like.

While various procedures involving the use of antibodies have been applied in the treatment of tumors, few if any successful attempts using activated cytotoxic T-cells have been recorded. Theoretically, cytotoxic T-cells would be the preferable means of treating tumors. However, no procedures have been available to specifically activate cytotoxic T-cells. In contrast to antibodies, the T-cell receptors on the surface of CD8 cells cannot recognize foreign antigens directly. Antigen must first be presented to the T cell receptor, such as a dendritic cell.

The presentation of antigen to CD8 T-cells is accomplished by major histocompatibility complex (MHC) molecules of the Class I type. The major histocompatibility complex (MHC) refers to a large genetic locus encoding an extensive family of glycoproteins which play an important role in the immune response. The MHC genes, which are also referred to as the HLA (human leukocyte antigen) complex, are located on chromosome 6 in humans. The molecules encoded by MHC genes are present on cell surfaces and are largely responsible for recognition of tissue transplants as "non-self". Thus, membrane-bound MHC molecules are intimately involved in recognition of antigens by T-cells.

MHC products are grouped into three major classes, referred to as I, II, and III. T-cells that serve mainly as helper cells express CD4 and primarily interact with Class II molecules, whereas CD8-expressing cells, which mostly represent cytotoxic effector

5 cells, interact with Class I molecules.

Class I molecules are membrane glycoproteins with the ability to bind peptides derived primarily from intracellular degradation of endogenous proteins. Complexes of MHC molecules with peptides derived from viral, bacterial and other foreign proteins

10 comprise the ligand that triggers the antigen responsiveness of T-cells. In contrast, complexes of MHC molecules with peptides derived from normal cellular products play a role in "teaching" the T-cells to tolerate self-peptides, in the thymus. Class I molecules do not present entire, intact antigens; rather, they present peptide fragments thereof, "loaded" onto their "peptide binding groove".

15

As will be recognized by those in the art, the term "host compatible" or "autologous" cells means cells that are of the same or similar haplotype as that of the subject or "host" to which the cells are administered.

20 The presentation of Class I MHC molecules bound to peptide alone has generally been ineffective in activating CD8 cells. In nature, the CD8 cells are activated by antigen-presenting cells, such as, for example, dendritic cells, which present not only a peptide-bound Class I MHC molecule, but also a costimulatory molecule. Such costimulatory molecules include B7 which is now recognized to be two subgroups

25 designated as B7.1 and B7.2, ICAM-1 and LFA-3. It has also been found that cell adhesion molecules such as integrins assist in this process.

Dendritic cells are antigen-presenting cells that are found in all tissues and organs, including the blood. Specifically, dendritic cells present antigens for T

30 lymphocytes, i.e., they process and present antigens, and stimulate responses from naive and memory T cells. In addition to their role in antigen presentation, dendritic cells

directly communicate with non-lymph tissue and survey non-lymph for an injury signal (e.g., ischemia, infection, or inflammation) or tumor growth. Once signaled, dendritic cells initiate the immune response by releasing IL-1 which triggers lymphocytes and monocytes. When the CD8 T-cell interacts with an antigen-presenting cell, such as a

5 dendritic cell, having the peptide bound by a Class I MHC and costimulatory molecule, the CD8 T-cell is activated to proliferate and becomes an effector T-cell. See, generally, Janeway and Travers, Immunobiology, published by Current Biology Limited, London (1994), incorporated by reference.

Accordingly, what is needed and which the present invention provides, is a

10 means to activate T-cells so that they proliferate, become cytotoxic for cells expressing the desired antigen, such as for example, MUC-1, and maintain memory cells specific for the administered antigen. Thus, the immune system is primed against various tumor epitopes so if spontaneous tumors arise, a pool of primed immune cells exist which become activated to recognize and kill the tumor cells.

15

Preferably, the epitopes presented to the immune system comprise agonist epitopes as described herein. Agonist polypeptides preferably comprise an amino acid sequence which is at least about 60% identical to the amino acid sequence of SEQ ID NO: 1 through 23, fragments or variants thereof, more preferably, the agonist

20 polypeptide comprises an amino acid sequence which is at least about 80% identical to the amino acid sequence of SEQ ID NO: 1 through 23. more preferably, the agonist polypeptide comprises an amino acid sequence which is at least about 90%, 95%, or 99.9% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

25 A review of the biology of memory T cells may be found in Dutton et al. (1998) *Ann. Rev Immunol* 16:201-23. Memory cells express a different pattern of cell surface markers, and they respond in several ways that are functionally different from those of naïve cells. Human memory cells are CD45RA⁻, CD45RO⁺. In contrast to naïve cells, memory cells secrete a full range of T cell cytokines.

Chemokines and cytokines also play a powerful role in the development of an immune response. The role of chemokines in leukocyte trafficking is reviewed by Baggiolini (1998) *Nature* 392:565-8, in which it is suggested that migration responses in the complicated trafficking of lymphocytes of different types and degrees of activation will be mediated by chemokines. The use of small molecules to block chemokines is reviewed by Baggiolini and Moser (1997) *J. Exp. Med.* 186:1189-1191.

The role of various specific chemokines in lymphocyte homing has been previously described. For example, Campbell et al. (1998) *Science*, showed that SDF-1 (also called PBSF), 6-C-kine (also called Exodus-2), and MIP-3beta (also called ELC or Exodus-3) induced adhesion of most circulating lymphocytes, including most CD4^{+} T cells; and MIP-3alpha (also called LARC or Exodus-1) triggered adhesion of memory, but not naïve, CD4^{+} T cells. Tangemann et al. (1998) *J. Immunol.* 161:6330-7 disclose the role of secondary lymphoid-tissue chemokine (SLC), a high endothelial venule (HEV)-associated chemokine, with the homing of lymphocytes to secondary lymphoid organs. Campbell et al. (1998) *J. Cell Biol* 141(4):1053-9 describe the receptor for SLC as CCR7, and that its ligand, SLC, can trigger rapid integrin-dependent arrest of lymphocytes rolling under physiological shear.

Mature B cells can be measured in immunoassays, for example, by cell surface antigens including CD19 and CD20 with monoclonal antibodies labeled with fluorochromes or enzymes may be used to these antigens. B cells that have differentiated into plasma cells can be enumerated by staining for intracellular immunoglobulins by direct immunofluorescence in fixed smears of cultured cells.

Several different ways, to assess maturity and cell differentiation, are available. For example, one such method is by measuring cell phenotypes. The phenotypes of immune cells and any phenotypic changes can be evaluated by flow cytometry after immunofluorescent staining using monoclonal antibodies that will bind membrane proteins characteristic of various immune cell types.

A second means of assessing cell differentiation is by measuring cell function. This may be done biochemically, by measuring the expression of enzymes, mRNA's,

genes, proteins, or other metabolites within the cell, or secreted from the cell. Bioassays may also be used to measure functional cell differentiation or measure specific antibody production directed at a patient's tumor, tumor cell lines or cells from fresh tumors.

Immune cells express a variety of cell surface molecules which can be detected with either monoclonal antibodies or polyclonal antisera. Immune cells that have undergone differentiation or activation can also be enumerated by staining for the presence of characteristic cell surface proteins by direct immunofluorescence in fixed smears of cultured cells.

In vitro T cell cytotoxic assays are well known to those skilled in the art. A preferred method is to measure cytotoxicity in a 5 hr ^{51}Sodium chromate (^{51}Cr) release assay. In particular, a 20 hr ^{51}Cr-release assay is preferred. Tumor cells, also referred to herein as "target cells" are plated in flat-bottomed microtiter plates and incubated at 37ºC overnight. The targets are washed and labeled the next day with ^{51}Cr at 37ºC. ^{51}Cr is taken up by the target cells, either by endocytosis or pinocytosis, and is retained in the cytoplasm. The wells containing tumor cells are washed, and then armed or unarmed ATC, referred to as "effector cells" are plated at different E:T ratios and incubated overnight at 37ºC. Cytolysis is a measure of the ^{51}Cr released from the target cells into the supernatant due to destruction of the target cells by the effector cells. The microtiter plates are centrifuged at 1000 rpm for 10 minutes and an aliquot of about 50 µl to about 100 µl is removed and the level of radioactivity is measured the next day by a gamma counter and the percent specific lysis calculated.

Percent specific lysis is measured by using the formula:

(^{51}Cr released from the target cells) – (spontaneous ^{51}Cr released from the target cells)/
(maximum ^{51}Cr released from the target cells) - (spontaneous ^{51}Cr released from the target cells) X
100

The spontaneous ^{51}Cr released from the target cells is measured with tumor cells to which no effector cells have been added. Maximum ^{51}Cr released from the target

cells is obtained by adding, for example, 1M HCl and represents the total amount of ^{51}Cr present in the cytoplasm of the target cell.

Other means of assaying for T lymphocyte activity is by the mixed lymphocyte reaction described in the examples which follow. Other cytotoxicity assays such as the labeling of target cells with tritiated thymidine (^{3}H-TdR) may also be used. ^{3}H-TdR is taken up by target cells into the nucleus of the cell. Release of ^{3}H-TdR is a measure of cell death by DNA fragmentation. The assay is conducted as above except the incubation period is at least about 48 hours and 50 µl to about 100 µl of the supernatant is measured by a beta-counter in the presence of at least about 1 ml of scintillation fluid. Calculation of percent specific lysis is performed using the above formula.

In a preferred embodiment the polypeptide is expressed at least at a higher level in a patient with cancer as compared to expression levels in normal individuals, preferably the polypeptide is expressed at least about 5 to about 10 fold higher in a patient with cancer as compared to expression in a normal individual. Preferably the cancer is a MUC-1$^+$ cancer and the subject sample is obtained from a mammalian patient, including a primate such as a human patient.

In another preferred embodiment, the invention provides for a method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising isolating dendritic cells from a patient suffering from cancer; and, treating the dendritic cells with one or more of the polypeptides identified by SEQ ID NO: 1 through 23; fragments and variants thereof. Preferably, the treated dendritic cells are administered to the patient.

In yet another preferred embodiment, autologous dendritic cells can be isolated from a patient, transduced with the vectors described in detail herein, cultured, and re-infused into the patient.

An "isolated" or "purified" population of cells is substantially free of cells and materials with which it is associated in nature. By substantially free or substantially

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purified APCs is meant at least 50% of the population are APCs, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of non-APCs cells with which they are associated in nature.

5   Dendritic cells of different maturation stages can be isolated based on the cell surface expression markers. For example, mature dendritic cells are less able to capture new proteins for presentation but are much better at stimulating resting T cells to grow and differentiate. Thus, mature dendritic cells can be of importance. Mature dendritic cells can be identified by their change in morphology; by their nonadherence; and by the

10   presence of various markers. Such markers include, but are not limited to, cell surface markers such as B7.2, CD40, CD11c$^+$, and MHC class II. Alternatively, maturation can be identified by observing or measuring the production of pro-inflammatory cytokines. Dendritic cells can be collected and analyzed using typical cytofluorography and cell sorting techniques and devices, such as a fluorescence-activated cell sorter (FACS).

15   Antibodies specific to cell surface antigens of different stages of dendritic cell maturation are commercially available.

        The amount of dendritic cells administered to the patient will also vary depending on the condition of the patient and should be determined via consideration of

20   all appropriate factors by the practitioner. Preferably, however, about $1x10^6$ to about $1x10^{12}$, more preferably about $1x10^8$ to about $1x10^{11}$, and even more preferably, about $1x10^9$ to about $1x10^{10}$ dendritic cells are utilized for adult humans. These amounts will vary depending on the age, weight, size, condition, sex of the patient, the type of tumor to be treated, the route of administration, whether the treatment is regional or systemic,

25   and other factors. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstance and needs of the patient.

        Methods of re-introducing cellular components are known in the art and include

30   procedures such as those exemplified in U.S. Pat. No. 4,844,893 to Honsik, *et al.* and U.S. Pat. No. 4,690,915 to Rosenberg. For example, administration of activated CD8

cells via intravenous infusion is appropriate. Any toxicity, from donor cell infusion, observed in a pregnant female will result in immediate cessation of any further infusions. Toxicity is measured according to the National Cancer Institute (NCI) scale.

5   *Toxicity Grading - The NCI Common Toxicity Scale.*

    *   If Grade I- II toxicities occur, the patient may continue with the infusion schedule.

    *   If Grade III toxicity occurs, the "drug" will be held until the toxicity decreases to Grade I or II, then the infusion will be restarted. If Grade III or IV toxicity

10 occurs after the restart, the "drug" infusions will be stopped.

    *   If Grade IV toxicity occurs, the patient is scored as having Grade IV toxicity and the next infusion is reduced to the previous dose. If the previous dose causes Grade IV toxicity, then the "drug" will be stopped.

    *   If Grade IV toxicity occurs in 1 of 3 patients at a specific dose level, an

15 additional 3 patients must be entered at that cell-dose level for a total of 6 patients at that dose level. If 2 of 6 patients at a cell-dose level develop Grade IV toxicity, this dose is defined as the maximum tolerated dose (MTD). The next 3 patients will be given 66% (two-thirds) of the previous cell-dose level. For the purposes of evaluation for dose-escalation, each patient at the same dose level should received at least 4 of 6

20 infusions.

    Large quantities of antigen-presenting dendritic cells can be generated *ex vivo* as described in U.S. Patent No: 6,497,876, which is incorporated herein, in its entirety. Following collection of an individual's CD34$^+$ hematopoietic progenitors and stem cells,

25 cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and flt-3 ligand (flt3-L) can be used to expand the cells *in vitro* and to drive them to differentiate into cells of the dendritic cell lineage. Cytokines can also be used to increase the numbers of CD34$^+$ cells in circulation prior to collection. The resulting dendritic cells are exposed to an antigen one wishes to elicit an immune response

30 against, and allowed to process the antigen (this procedure is sometimes referred to in the art as "antigen-pulsing"). The antigen-pulsed (or antigen-expressing) dendritic cells

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are then activated with a CD40 binding protein, and subsequently administered to the individual.

Dendritic cells comprise a heterogeneous cell population with distinctive
5   morphology and a widespread tissue distribution. The dendritic cell system and its role
in immunity is reviewed by Steinman, R. M., *Annu. Rev. Immunol.*, 9:271-296 (1991),
incorporated herein by reference. The cell surface of dendritic cells is unusual, with
characteristic veil-like projections, and is characterized by having the cell surface
markers CD1a$^+$, CD4$^+$, CD86$^+$, or HLA-DR$^+$. Dendritic cells have a high capacity for
10 sensitizing MHC-restricted T cells and are very effective at presenting antigens to T cells
in situ, both self-antigens during T cell development and tolerance and foreign antigens
during immunity.

Because of their effectiveness at antigen presentation, autologous dendritic cells
15 preferably are used *ex vivo* as alloantigen adjuvants (see, for example, Romani, et al., *J.
Exp. Med.*, 180:83 (1994). The use of dendritic cells as immunostimulatory agents has
been limited due to the low frequency of dendritic cells in peripheral blood, the limited
accessibility of lymphoid organs and the dendritic cells' terminal state of differentiation.
Dendritic cells originate from CD34$^+$ bone marrow or peripheral blood progenitors and
20 peripheral blood mononuclear cells, and the proliferation and maturation of dendritic
cells can be enhanced by the cytokines GM-CSF sargramostim, Leukine™ (Immunex
Corporation, Seattle, Wash.), TNF-α, c-kit ligand (also known as stem cell factor (SCF),
steel factor (SF), or mast cell growth factor (MGF)) and interleukin-4. Recently, flt3-L
has been found to stimulate the generation of large numbers of functionally mature
25 dendritic cells, both *in vivo* and *in vitro*.

Ex Vivo Culture of Dendritic Cells

A procedure for *ex vivo* expansion of hematopoietic stem and progenitor cells is
described in U.S. Pat. No. 5,199,942, incorporated herein by reference. Other suitable
30 methods are known in the art. Briefly, *ex vivo* culture and expansion comprises: (1)
collecting CD34$^+$ hematopoietic stem and progenitor cells from a patient from peripheral

blood harvest or bone marrow explants; and (2) expanding such cells *ex vivo*. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used.

5   Stem or progenitor cells having the CD34 marker constitute only about 1% to 3% of the mononuclear cells in the bone marrow. The amount of $CD34^+$ stem or progenitor cells in the peripheral blood is approximately 10- to 100-fold less than in bone marrow. Cytokines such as flt3-L may be used to increase or mobilize the numbers of dendritic cells *in vivo*. Increasing the quantity of an individual's dendritic cells may facilitate

10   antigen presentation to T cells for antigen(s) that already exists within the patient, such as a tumor antigen, or a bacterial or viral antigen. Alternatively, cytokines may be administered prior to, concurrently with or subsequent to administration of an antigen to an individual for immunization purposes.

15   Peripheral blood cells are collected using apheresis procedures known in the art. See, for example, Bishop et al., *Blood*, vol. 83, No. 2, pp. 610-616 (1994). Briefly, peripheral blood progenitor cells (PBPC) and peripheral blood stem cells (PBSC) are collected using conventional devices, for example, a Haemonetics Model V50 apheresis device (Haemonetics, Braintree, Mass.). Four-hour collections are performed typically

20   no more than five times weekly until approximately 6.5×10^8 mononuclear cells (MNC)/kg are collected. The cells are suspended in standard media and then centrifuged to remove red blood cells and neutrophils. Cells located at the interface between the two phases (the buffy coat) are withdrawn and resuspended in HBSS. The suspended cells are predominantly mononuclear and a substantial portion of the cell mixture are early

25   stem cells.

A variety of cell selection techniques are known for identifying and separating $CD34^+$ hematopoietic stem or progenitor cells from a population of cells. For example, monoclonal antibodies (or other specific cell binding proteins) can be used to bind to a

30   marker protein or surface antigen protein found on stem or progenitor cells. Several

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such markers or cell surface antigens for hematopoietic stem cells (i.e., flt-3, CD34, My-10, and Thy-1) are known in the art, as are specific binding proteins.

In one method, antibodies or binding proteins are fixed to a surface, for example, glass beads or flask, magnetic beads, or a suitable chromatography resin, and contacted with the population of cells. The stem cells are then bound to the bead matrix. Alternatively, the binding proteins can be incubated with the cell mixture and the resulting combination contacted with a surface having an affinity for the antibody-cell complex. Undesired cells and cell matter are removed providing a relatively pure population of stem cells. The specific cell binding proteins can also be labeled with a fluorescent label, e.g., chromophore or fluorophore, and the labeled cells separated by sorting. Preferably, isolation is accomplished by an immunoaffinity column.

Immunoaffinity columns can take any form, but usually comprise a packed bed reactor. The packed bed in these bioreactors is preferably made of a porous material having a substantially uniform coating of a substrate. The porous material, which provides a high surface area-to-volume ratio, allows for the cell mixture to flow over a large contact area while not impeding the flow of cells out of the bed. The substrate should, either by its own properties, or by the addition of a chemical moiety, display high-affinity for a moiety found on the cell-binding protein. Typical substrates include avidin and streptavidin, while other conventional substrates can be used.

In one useful method, monoclonal antibodies that recognize a cell surface antigen on the cells to be separated are typically further modified to present a biotin moiety. The affinity of biotin for avidin thereby removably secures the monoclonal antibody to the surface of a packed bed (see Berenson, et al., *J. Immunol. Meth.*, 91:11, 1986). The packed bed is washed to remove unbound material, and target cells are released using conventional methods. Immunoaffinity columns of the type described above that utilize biotinylated anti-CD34 monoclonal antibodies secured to an avidin-coated packed bed are described for example, in WO 93/08268.

An alternative means of selecting the quiescent stem cells is to induce cell death in the dividing, more lineage-committed, cell types using an antimetabolite such as 5-fluorouracil (5-FU) or an alkylating, agent such as 4-hydroxycyclophosphamide (4-HC). The non-quiescent cells are stimulated to proliferate and differentiate by the addition of

5   growth factors that have little or no effect on the stem cells, causing the non-stem cells to proliferate and differentiate and making them more vulnerable to the cytotoxic effects of 5-FU or 4-HC. See Berardi et al., *Science*, 267:104 (1995), which is incorporated herein by reference.

10   Isolated stem cells can be frozen in a controlled rate freezer (e.g., Cryo-Med, Mt. Clemens, Mich.), then stored in the vapor phase of liquid nitrogen using dimethylsulfoxide as a cryoprotectant. A variety of growth and culture media can be used for the growth and culture of dendritic cells (fresh or frozen), including serum-depleted or serum-based media. Useful growth media include RPMI, TC 199, Iscoves

15   modified Dulbecco's medium (Iscove, et al., F. *J. Exp. Med.*, 147:923 (1978)), DMEM, Fischer's, alpha medium, NCTC, F-10, Leibovitz's L-15, MEM and McCoy's. Particular nutrients present in the media include serum albumin, transferrin, lipids, cholesterol, a reducing agent such as 2-mercaptoethanol or monothioglycerol, pyruvate, butyrate, and a glucocorticoid such as hydrocortisone 2-hemisuccinate. More particularly, the standard

20   media includes an energy source, vitamins or other cell-supporting organic compounds, a buffer such as HEPES, or Tris, that acts to stabilize the pH of the media, and various inorganic salts. A variety of serum-free cellular growth media is described in WO 95/00632, which is incorporated herein by reference. The collected CD34$^+$ cells are cultured with suitable cytokines, for example, as described herein. CD34$^+$ cells then are

25   allowed to differentiate and commit to cells of the dendritic lineage. These cells are then further purified by flow cytometry or similar means, using markers characteristic of dendritic cells, such as CD1a, HLA DR, CD80 and/or CD86. The cultured dendritic cells are exposed to an antigen, for example, an allogeneic class I HLA molecule, allowed to process the antigen, and then cultured with an amount of a CD40 binding

30   protein to activate the dendritic cell. Alternatively, the dendritic cells are transfected with a gene encoding an allogeneic HLA class I molecule or immune related receptors,

and then cultured with an amount of a CD40 binding protein to activate the antigen-presenting dendritic cells.

   The activated, antigen-carrying dendritic cells are then administered to an
5 individual in order to stimulate an antigen-specific immune response. The dendritic cells
   can be administered prior to, concurrently with, or subsequent to, antigen administration.
   Alternatively, T cells may be collected from the individual and exposed to the activated,
   antigen-carrying dendritic cells *in vitro* to stimulate antigen-specific T cells, which are
   administered to the individual.
10

Useful Cytokines

   Various cytokines will be useful in the *ex vivo* culture of dendritic cells. Flt3-L
   refers to a genus of polypeptides that are described in EP 0627487 A2 and in WO
   94/28391, both incorporated herein by reference. A human flt3-L cDNA was deposited
15 with the American Type Culture Collection, Rockville, Md., USA (ATCC) on Aug. 6,
   1993 and assigned accession number ATCC 69382. IL-3 refers to a genus of
   interleukin-3 polypeptides as described in U.S. Pat. No. 5,108,910, incorporated herein
   by reference. A DNA sequence encoding human IL-3 protein suitable for use in the
   invention is publicly available from the American Type Culture Collection (ATCC)
20 under accession number ATCC 67747. c-kit ligand is also referred to as Mast Cell
   Growth Factor (MGF), Steel Factor or Stem Cell Factor (SCF), and is described in EP
   423,980, which is incorporated herein by reference. Other useful cytokines include
   Interleukin-4 (IL-4; Mosley et al., *Cell* 59:335 (1989), Idzerda et al., *J. Exp. Med.*
   171:861 (1990) and Galizzi et al., *Intl. Immunol.* 2:669 (1990), each of which is
25 incorporated herein by reference) and granulocyte-macrophage colony stimulating factor
   (GM-CSF; described in U.S. Pat. Nos. 5,108,910, and 5,229,496 each of which is
   incorporated herein by reference). Commercially available GM-CSF (sargramostim,
   Leukine™) is obtainable from Immunex Corp., Seattle, Wash.). Moreover, GM-
   CSF/IL-3 fusion proteins (i.e., a C-terminal to N-terminal fusion of GM-CSF and IL-3)
30 will also be useful in *ex vivo* culture of dendritic cells. Such fusion proteins are known
   and are described in U.S. Pat. Nos. 5,199,942, 5,108,910 and 5,073,627, each of which is

incorporated herein by reference. A preferred fusion protein is PIXY321 as described in
U.S. Pat. No. 5,199,942.

Useful cytokines act by binding a receptor present on the surface of a dendritic
cell and transducing a signal. Moreover, additional binding proteins can be prepared as
described herein for CD40 binding proteins, that bind appropriate cytokine receptors and
transduce a signal to a dendritic cell. For example, WO 95/27062 describes agonistic
antibodies to Flt-3, the receptor for Flt-3L, from which various Flt-3 binding. proteins
can be prepared. Additional useful cytokines include biologically active analogs of
cytokines that are useful for culturing dendritic cells. Useful cytokine analogs have an
amino acid sequence that is substantially similar to the native cytokine, and are
biologically active capable of binding to their specific receptor and transducing a
biological signal. Such analogs can be prepared and tested by methods that are known in
the art.

An alternate method for preparing dendritic cells that present antigen is to
transfect the dendritic cells with a gene encoding an antigen or a specific polypeptide
derived therefrom. Once the dendritic cells express the antigen in the context of MHC,
the dendritic cells are activated with a CD40 binding protein, and subsequently
administered to the individual to provide a stronger and improved immune response to
the antigen.

The activated antigen-presenting dendritic cells can also be used as a vaccine
adjuvant and can be administered prior to, concurrently with or subsequent to antigen
administration. Moreover, the dendritic cells can be administered to the individual prior
to, concurrently with or subsequent to administration of cytokines that modulate an
immune response, for example a CD40 binding protein (i.e., soluble CD40L), or a
soluble CD83 molecule. Additional useful cytokines include, but are not limited to,
Interleukins (IL) 1, 2, 4, 5, 6, 7, 10, 12 and 15, colony stimulating factors (CSF) such as
GM-CSF, granulocyte colony stimulating factor (G-CSF), or GM-CSF/IL-3 fusion

proteins, or other cytokines such as TNF-α or c-kit ligand. Moreover, biologically active derivatives of these cytokines; and combinations thereof will also be useful.

CD40 is a member of the tumor necrosis factor (TNF)/nerve growth factor
5 (NGF) receptor family, which is defined by the presence of cysteine-rich motifs in the extracellular region (Smith et al., *Science* 248:1019, 1990; Mallett and Barclay, *Immunology Today* 12:220; 1991). This family includes the lymphocyte antigen CD27, CD30 (an antigen found on Hodgkin's lymphoma and Reed-Stemberg cells), two receptors for TNF, a murine protein referred to as 4-1BB, rat OX40 antigen, NGF
10 receptor, and Fas antigen. Human CD40 antigen (CD40) is a peptide of 277 amino acids having a molecular weight of 30,600 (Stamenkovic et al., *EMBO J.* 8:1403, 1989). CD40L is believed to be important in feedback regulation of an immune response. For example, a CD40$^+$ antigen presenting cell will present antigen to a T cell, which will then become activated and express CD40L. The CD40L will, in turn, further activate the
15 antigen presenting cell, increasing its efficiency at antigen presentation, and upregulating expression of Class I and Class II MHC, CD80 and CD86 costimulatory molecules, as well as various cytokines (Caux et al., *J. Exp. Med.* 180:1263, 1994).

Purified dendritic cells are then pulsed with (exposed to) antigen, to allow them
20 to take up the antigen in a manner suitable for presentation to other cells of the immune systems. Antigens are classically processed and presented through two pathways. Peptides derived from proteins in the cytosolic compartment are presented in the context of Class I MHC molecules, whereas peptides derived from proteins that are found in the endocytic pathway are presented in the context of Class II MHC. However, those of
25 skill in the art recognize that there are exceptions; for example, the response of CD8$^+$ tumor specific T cells, which recognize exogenous tumor antigens expressed on MHC Class I. A review of MHC-dependent antigen processing and peptide presentation is found in Germain, R. N., *Cell* 76:287 (1994).

30 Numerous methods of pulsing dendritic cells with antigen are known; those of skill in the art regard development of suitable methods for a selected antigen as routine

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experimentation. In general, the antigen is added to cultured dendritic cells under conditions promoting viability of the cells, and the cells are then allowed sufficient time to take up and process the antigen, and express antigen peptides on the cell surface in association with either Class I or Class II MHC, a period of about 24 hours (from about

5   18 to about 30 hours, preferably 24 hours). Dendritic cells may also be exposed to antigen by transfecting them with DNA encoding the antigen. The DNA is expressed, and the antigen is presumably processed via the cytosolic/Class I pathway.

The present invention provides methods of using therapeutic compositions
10   comprising activated, antigen-pulsed dendritic cells. The use of such cells in conjunction with soluble cytokine receptors or cytokines, or other immunoregulatory molecules is also contemplated. The inventive compositions are administered to stimulate an allogeneic immune response, and can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique.
15   Typically, the cells on the will be administered in the form of a composition comprising the antigen-pulsed, activated dendritic cells in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate diluents.

20

For use in stimulating a certain type of immune response, administration of other cytokines along with activated, antigen-pulsed dendritic cells is also contemplated. Several useful cytokines (or peptide regulatory factors) are discussed in Schrader, J. W. (*Mol Immunol* 28:295; 1991). Such factors include (alone or in combination)
25   Interleukins 1, 2, 4, 5, 6, 7, 10, 12 and 15; granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor; a fusion protein comprising Interleukin-3 and granulocyte-macrophage colony stimulating factor; Interferon-γ, TNF, TGF-β, flt-3 ligand and biologically active derivatives thereof. A particularly preferred cytokine is CD40 ligand (CD40L). Other cytokines will also be useful, as described herein. DNA
30   encoding such cytokines will also be useful in the inventive methods, for example, by transfecting the dendritic cells to express the cytokines. Administration of these

immunomodulatory molecules includes simultaneous, separate or sequential administration with the cells of the present invention.

In another preferred embodiment, the invention provides for a polypeptide identified by any one of SEQ ID NO: 1 through 23 having a homology to anyone one of SEQ ID NO: 1 through 23 of at least about 10%, more preferably, 25%, even more preferably about 40%, 50%, 60%, 70%, 80%, 90%, or 99.9%. Dendritic cells can be pulsed with any of these polypeptides during *ex-vivo* culture.

In one aspect of the invention, the polypeptide comprises SEQ ID NO: 2. Preferably, the polypeptide binds to HLA molecules with a high avidity and has a higher association constant (K_a) for the HLA than a native polypeptide and/or a lower dissociation constant (K_d) for the HLA than a native polypeptide.

In another aspect of the invention, the polypeptide is derived from a mucin tumor antigen, preferably, the polypeptide is derived from a non-variable number of tandem repeats region of MUC-1.

In another aspect of the invention, antigen presentation, by antigen presenting cells of the polypeptides induces an immune response, preferably a cellular immune response. For example, the cellular immune response is a cytotoxic T cell response, a T helper cell response, or a B cell immune response.

In yet another aspect, variants of the nucleic acid molecule encoding polypeptides as identified by SEQ ID NO: 1 through 23 can be used to transduce immune cells for the detection and lysing of, for example, MUC-1 positive cancers. An "allele" or " variant" is an alternative form of a gene. Of particular utility in the invention are variants of the genes encoding any potential MUC-1^{+} tumor cell markers identified by the methods of this invention. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural

or recombinant gene may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

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The compositions and methods of the present invention also encompass variants of the above polypeptides and nucleic acid sequences encoding such polypeptides. A polypeptide "variant," as used herein, is a polypeptide that differs from the native polypeptide in substitutions and/or modifications, such that the antigenic and/or

10 immunogenic properties of the polypeptide are retained. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antisera and/or T-cells as described above. Nucleic acid variants may contain one or more substitutions, deletions, insertions and/or modifications such that the antigenic and/or immunogenic properties of the encoded

15 polypeptide are retained. One preferred variant of the polypeptides described herein is a variant that contains nucleotide substitutions, deletions, insertions and/or modifications at no more than 20% of the nucleotide positions.

Preferably, but not limited to, a variant contains conservative substitutions. A
20 "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys,
25 ser, tyr, thr; (3) vat, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. However, any type of substitution is within the scope and embodiments of the invention.

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenic or antigenic
30 properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end

of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may
5   be conjugated to an immunoglobulin Fc region.

In general, nucleotide sequences encoding all or a portion of the polypeptides described herein may be prepared using any of several techniques. For example, cDNA molecules encoding such polypeptides may be cloned on the basis of the MUC-1 tumor-
10   specific expression of the corresponding mRNAs, using differential display PCR. This technique compares the amplified products from RNA template prepared from normal and MUC-1 positive tumor tissue. cDNA may be prepared by reverse transcription of RNA using a random primer, such as for example, $(dT)_{12}$ AG primer. Following amplification of the cDNA using a random primer, a band corresponding to an amplified
15   product specific to the tumor RNA may be cut out from a silver stained gel and subcloned into a suitable vector, such as the adenovirus vector described in the examples which follow. Nucleotide sequences encoding all or a portion of the MUC-1 tumor-specific polypeptides disclosed by any one of SEQ ID NOs:1 through 6 and variants thereof may be amplified from cDNA prepared as described above using any random
20   primers.

Alternatively, a gene encoding a polypeptide as described herein (or a portion thereof) may be amplified from human genomic DNA, or from tumor cell cDNA, via polymerase chain reaction.
25

In an embodiment of the invention the presence of the one or more nucleic acid molecules is correlated to a sample of a normal subject. The sample is preferably obtained from a mammal suspected of having a proliferative cell growth disorder, in particular, a MUC-1^{+} cancer.
30

Percent identity and similarity between two sequences (nucleic acid or polypeptide) can be determined using a mathematical algorithm (see, e.g., *Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed.,

5   Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

10   In another preferred embodiment, MUC-1 peptide fragments and derivatives of the invention are of a sufficient length such that they activate the immune system resulting in the lysing of cancer cells, such as, for example cells expressing MUC-1. MUC-1 nucleic acid molecules, fragments and derivatives encoding for any one of the polypeptides identified by SEQ ID NO: 1 through 23, thus preferably comprise at least

15   about 90% nucleotides as compared to the sequence identified by any one of SEQ ID NO: 1 through 23, usually at least about 80% nucleotides as compared to the sequence identified by any one of SEQ ID NO: 1 through 23, more usually at least about 70% nucleotides as compared to the sequence identified by anyone of SEQ ID NO: 1 through 23, even more typically at least about 40% or 50% nucleotides.

20

To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred

25   embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the nucleotide as the

30   corresponding position in the second sequence, then the molecules are identical at that position (as used herein nucleic acid "identity" is equivalent to nucleic acid

"homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

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The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at online through the Genetics
10   Computer Group), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A preferred, non-limiting example of parameters to be used in conjunction with the GAP program include a Blosum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

15

In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of Meyers and Miller (Comput. Appl. Biosci. 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0 or version 2.0U), using a PAM120 weight residue table, a gap length
20   penalty of 12 and a gap penalty of 4.

The treatment of neoplastic disease or neoplastic cells, refers to an amount of the vectors and/or peptides, described throughout the specification and in the Examples which follow, capable of invoking one or more of the following effects: (1) inhibition, to
25   some extent, of tumor growth, including, (i) slowing down and (ii) complete growth arrest; (2) reduction in the number of tumor cells; (3) maintaining tumor size; (4) reduction in tumor size; (5) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention, of tumor cell infiltration into peripheral organs; (6) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention, of metastasis; (7)
30   enhancement of anti-tumor immune response, which may result in (i) maintaining tumor size, (ii) reducing tumor size, (iii) slowing the growth of a tumor, (iv) reducing, slowing

or preventing invasion or (v) reducing, slowing or preventing metastasis; and/or (8) relief, to some extent, of one or more symptoms associated with the disorder.

Thus in one aspect of the invention any variant, fragment, mutant can be used to transduce immune cells, such as for example dendritic cells, for the treatment of an individual suffering from, or, prophylactically to an individual susceptible to cancer. As discussed above, a preferred use of nucleic acid sequences identified in the present invention, is for the generation of treatments that lyse for example, MUC-1 cancer cells. The nucleic acid molecules can be expressed by a vector containing a DNA segment encoding the wild-type, alleles, variants, mutations or fragments of the genes. Mutations and alleles of the nucleic acid molecules are also preferably used in the construction of a vector for use in treatment. The vector comprising the desired nucleic acid sequence for conferring resistance to, for example, MUC-1 positive cancer, preferably has at least one such nucleic acid sequence. Alternatively, the vector may be comprised of more than one such nucleic acid sequence, or combinations of allelic variants. The vector can also be comprised of cassettes of different allelic variants or wild type nucleic acid molecules.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (herein "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D. N. Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed. 1984); Nucleic Acid Hybridization [B. D. Hames & S. J. Higgins eds. (1985)]; Transcription And Translation [B. D. Hames & S. J. Higgins, eds. (1984)]; Animal Cell Culture [R. I. Freshney, ed. (1986)]; Immobilized Cells And Enzymes [IRL Press, (1986)]; B. Perbal, A Practical Guide To Molecular Cloning (1984); F. M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Introducing the genes, fragments or alleles thereof, into an individual can include use of vectors, liposomes, naked DNA, adjuvant-assisted DNA, gene gun, catheters, etc. Vectors include chemical conjugates such as described in WO 93/04701, which has a targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage etc. The vectors can be chromosomal, non-chromosomal or synthetic.

In another preferred embodiment, cells are isolated and purified cell from a sample, patient or donor individual and are used in functional assays to determine any properties of the cells. Depending on the isolated and purified cellular population, appropriate functional assays known in the art can be conducted. For example, if the population of cells are T cells specific for a desired antigen such as a tumor antigen, cytotoxic T cell assays, T cell proliferation assays, cytokine profiles, determination of surface antigens for T cell maturity or memory T cells, etc., can be carried out.

Isolation of cells useful in the present invention are well known in the art. For example, peripheral blood mononuclear cells (PBMCs) can be obtained from a subject and isolated by density gradient centrifugation, e.g., with Ficoll/Hypaque. Specific cell populations can be depleted or enriched using standard methods. For example, monocytes/macrophages can be isolated by adherence on plastic. T cells or B cells can be enriched or depleted, for example, by positive and/or negative selection using antibodies to T cell or B cell surface markers, for example by incubating cells with a specific primary monoclonal antibody (mAb), followed by isolation of cells that bind the mAb using magnetic beads coated with a secondary antibody that binds the primary mAb. Peripheral blood or bone marrow derived hematopoietic stem cells can be isolated by similar techniques using stem cell-specific mAbs (e.g., anti-CD34 mAbs). Specific cell populations can also be isolated by fluorescence activated cell sorting according to

standard methods. Monoclonal antibodies to cell-specific surface markers known in the art and many are commercially available.

If desired, a large proportion of terminally differentiated cells may be removed by initially using a "relatively crude" separation. For example, magnetic bead separations may be used initially to remove large numbers of lineage committed cells. Desirably, at least about 80%, usually at least 70% of the total hematopoietic cells can be removed.

Procedures for separation may include but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique.

Techniques providing accurate separation include but are not limited to, flow cytometry, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention. The following non-limiting examples are illustrative of the invention.

EXAMPLES

Materials and Methods

Cell Cultures.

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The human breast adenocarcinoma cell line MCF-7 (HLA-A2 positive and MUC-1 positive), and SK-Mel-24 (HLA-A2 positive, MUC-1 negative) were purchased from American Type Culture Collection (Manassas, VA). The cultures were free of mycoplasma and were maintained in complete medium [RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies, Inc.)]. The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or B antigens. C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1. These cells were obtained from Dr. William E. Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). The 174CEM-T2 cell line (T2) transport deletion mutant was provided by Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT). C1R-A2 cells and T2 cells were mycoplasma free and were maintained in RPMI 1640 complete medium and in Iscove's modified Dulbecco's complete medium (Invitrogen Life Technologies), respectively.

Peptides.

The amino acid sequence of MUC-1 was scanned for matches to consensus motifs for HLA-A2 binding peptides. The computer algorithm from the BioInformatics and Molecule Analysis Section of NIH (BIMAS) that was developed by Parker K. C. *et al.*,(*J. Immunol.*, 152: 163-175, 1994) was used, which ranks potential MHC binding peptides according to the predictive one-half-time dissociation of peptide/MHC complexes. The HLA-A2 allele was chosen because they are the most commonly expressed class I allele. Ten-mer peptides from the non-variable number of tandem repeat sequence were synthesized if they conformed to the respective consensus motif. A panel of 10-mer MUC-1 peptides (Table 1) and analogues with single amino acid substitution to positions P1 to P10 of P-92 MUC-1 peptide (see Figure 1) were made by American Peptide Company (Sunnyvale, CA) with purity >90%. In addition, a CEA peptide CAP1-6D (28) >96% pure was made by American Peptide Company (Sunnyvale, CA).

Flow cytometric analysis.

(i) Single-color flow cytometric analysis.

The method for single-color flow cytometric analysis has been described previously (Gaudagni F. *et al. Cancer Res.*:50: 6248-6255, 1990.). Briefly, cells were washed three times with cold Ca^{2+} and Mg^{2+}-free DPBS and then stained for 1 h at 4°C using 1 μg of the mAb against HLA-A2 (A2, 28, One Lambda, Inc., Canoga Park, CA), CD3, CD4, CD8, and CD56 (BD Biosciences, San Jose, CA). Mineral oil plasmacytoma-104E (Cappel/Organon Teknika Corp., West Chester, PA) was used as an isotype control. The cells were then washed three times and incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (IgG) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The cells were immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm. Data were gathered from 10,000 live cells, stored and used to generate results.

(ii) Dual-color flow cytometric analysis.

The procedure for dual-color flow cytometric analysis was similar to that for single-color analysis with the following exceptions. Anti –MHC-class II FITC/ anti-CD11c PE, anti-MHC-class II FITC/anti-CD80 PE, anti-CD58 FITC/anti-CD54 PE, anti-MHC class I FITC/anti-MHC class II PE, and anti-IgG1 FITC/anti-IgG2a PE (isotype controls) were used for the analysis of dendritic cells; >96% of the dendritic cells were CD11c and MHC class II positive.

The antibodies used for the analysis of T cell lines were anti-CD56 FITC/anti-CD8 PE, anti-CD4 FITC/anti-CD8 PE and anti-CD45R0-FITC/anti-CD49d PE; >98% of the T-1191-P92 and T-1191-P-93L cells were CD8 positive. Antibodies to CD4, CD8, CD28, CD45RO, CD56, CD49d, CD54, CD80, CD86, CD58 and CD11c were purchased from BD Biosciences. Antibodies to MHC-class I and MHC-class II were purchased from Serotec, Oxford, UK. Staining was done simultaneously for 1 h, after which cells were washed three times, resuspended as above, and immediately analyzed

using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 mW at 488 nm with the use of the CELLQuest program.

Results were expressed in % of positive cells and mean fluorescence intensity 5 (MFI). MFI was used to express the levels of fluorescence determined by measuring the average for all the cells in the gated fluorescence dot plot. The MFI value was collected in log scale on the FACSCAN.

Peptide Binding to HLA-A2.

10 Binding of P-92 and P-92 analogues to HLA-A2 molecules was evaluated by binding to T2A2 cells as demonstrated by flow cytometry. In this assay, increased stability (accumulation) of HLA-A2 molecules on the surface of T2 cells as a consequence of peptide binding is measured by increased binding of antibody directed against HLA-A2 molecule. Briefly, 1 x 10^6 cells in serum-free Iscove's modified 15 Dulbecco's complete medium were incubated with peptides at a concentration of 50 μg/ml in 24-well culture plates at 37° C in 5% CO_2. Flow cytometry for peptide binding was performed using T2 cells and single-color analysis. After cells were washed three times in DPBS, as described above, they were incubated for 1 h with a 1:100 dilution of HLA-A2–specific MAb (One Lambda, Inc.) per 10^6 cells. UPC-10 (Cappel/Organon 20 Teknika) was used as isotype control. The cells were then washed three times and incubated with 1:100 dilution of FITC-labeled anti-mouse IgG (BD Biosciences). Analysis was conducted with the FACScan, as described above. Cells were maintained on ice during all cell preparation and staining.

25 *Culture of DCs from PBMCs.*

HLA-A2 normal donor PBMCs were obtained from heparinized blood. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC), as described previously (Boyum, A. *Scand. J. Clin. Lab. Invest.* 97(Suppl): 51-76, 1968.). DCs were prepared using a modification of the procedure 30 described by Sallusto *et al.* (*J. Exp. Med.*, 179: 1109-1118, 1994.). PBMCs (1.5 x 10^8) were resuspended in AIM-V medium containing 2 mM glutamine, 50 μg/ml

streptomycin, and 10 µg/ml gentamycin (Invitrogen Life Technologies, Inc.), and allowed to adhere to a T-150 flask (Corning Costar Corp., Cambridge, MA). After 2 h at 37° C, the non-adherent cells were removed with a gentle rinse. The adherent cells were cultured for 6-7 days in AIM-V medium containing 100ng/ml of recombinant human

5   GM-CSF (rhGM-CSF) and 20ng/ml of recombinant human IL-4 (rhIL-4). The culture medium was replenished every 3 days.

Recombinant Virus and Infection of DCs with Avipox Virus Containing MUC-1 (rF-MUC-1/TRICOM).

10   Recombinant fowlpox virus was constructed as described by Jenkins S., *et al.*, *AIDS Res. Hum. Retroviruses*, 7: 991-998, 1991. A plaque-purified isolate from the POXVAC-TC vaccine strain of fowlpox virus was used as the parental virus for this recombinant virus. The MUC-1, LFA-3, ICAM-1, and B7-1 sequences were inserted into the *Bam*HI, J region of the fowlpox virus genome. In addition, the *lacZ* gene, under

15   the control of the fowlpox C1 promoter, was included to identify and isolate recombinant viruses using a chromogenic assay for β-galactosidase. The MUC-1 gene that was inserted into fowlpox virus varies from the native MUC-1 gene. It encodes a 30-amino acid signal sequence, followed by the first 38 amino acids of the mature N-terminal sequence of the MUC-1 protein, 10 identical copies of the 20-amino acid

20   repeated sequence, and the C-terminal portion of the protein. The 600 bp repeated sequence was produced using overlapping synthetic oligonucleotides containing codons with numerous third-base variations in each repeat sequence without changing the encoded amino acids. This was done to minimize duplicated nucleotide sequences, which are unstable in pox viruses, while maintaining the repeated amino acid sequences.

25

    rF-MUC-1/TRICOM is a recombinant fowlpox virus that contains the MUC-1 gene under the control of the 40K promoter , the human LFA-3 gene under the control of the vaccinia 30K promoter , the human ICAM-1 gene under the control of the vaccinia I3 promoter, and the human B7-1 gene under the control of the synthetic early/late

30   (sE/L) promoter. Dendritic cells (DCs) (1 x 10^6) were incubated in 1 ml of Opti-MEM medium (Life Technologies, Inc.) at 37° C with rF-MUC-1/TRICOM or control avipox

virus vector (FP-WT). Titration experiments demonstrated that 4 x 10^7 plaque-forming units/ml, equal to an MOI of 40:1 for 2 h, were able to consistently induce transgene expression in approximately 75% of the infected DCs. The infected DCs were suspended in 10 ml of fresh, warm RPMI-1640 complete medium containing 100ng/ml

5   of rhGM-CSF, 20ng/ml rhIL-4, and 20ng/ml of TNF-α cultured for 24 h, and then subsequently used as APCs.

Generation of T-cell Lines.

Modification of the protocol described by Tsang K. Y. *et al.,* J. *Natl. Cancer*
10   *Inst.,* 87: 982-990, 1995, was used to generate MUC-1-specific CTL. To generate T-cell lines T-1191-P-93L and T-1191-P-92, autologous DCs infected with rF-MUC-1/TRICOM were used as APCs. Autologous non-adherent cells were then added to APCs at an effector-to-APC ratio of 10:1. Cultures were then incubated for 3 days at 37o C in a humidified atmosphere containing 5% CO2. The cultures were then supplemented
15   with recombinant human IL-2 at a concentration of 20 units/ml for 7 days; the IL-2 containing medium was replenished every 3 days. The 3-day incubation with peptide and 7-day IL-2 supplement constituted one IVS cycle. Primary cultures were restimulated with rF-MUC-1/TRICOM–infected autologous DCs as described above on day 11 to begin the next IVS cycle. rF-MUC-1/TRICOM–infected autologous DCs were used as
20   APCs for three IVS cycles. Irradiated (23,000 rads) autologous EBV-transformed B cells were used as APCs after the third IVS cycle. For the restimulation with EBV-transformed B cells, peptides at a concentration of 50 mg/ml were used to pulse autologous EBV-transformed B cells at a ratio of effector-to-APC of 1:3 for restimulation. Cultures were then incubated for 3 days at 37° C in a humidified
25   atmosphere containing 5% CO2. After removal of the peptide containing medium, the cultures were then supplemented with recombinant human IL-2 at a concentration of 20 unit/ml for days. T cell lines from patients 18 and 23 (T-18-P-92, T-18-P93L, T-23-P-92 and T-23-P93L) were generated by stimulation of PBMCs with autologous DCs pulsed with the P-92 or P93L peptides using the same stimulation protocol as described above.
30   The markers used for the analysis and identification of DCs were CD11c, MHC-class II, CD80, CD54, CD58 and CD83. CD3 was also used as a negative marker.

Cytotoxic Assay.

Target cells (C1R-A2 or tumor cells) were labeled with 50 μCi of [111]Indium-labeled oxyquinoline (Medi-Physics Inc., Arlington, IL) for 15 min at room temperature. Target cells (0.3×10^4) in 100 μl of RPMI-1640 complete medium were added to each of 96 wells in flat-bottomed assay plates (Corning Costar Corp.). Labeled C1R-A2 target cells were incubated with peptides at the concentration indicated for 60 min at 37° C in 5% CO_2 before adding effector cells. No peptide was used when carcinoma cell lines were used as targets. Effector cells were suspended in 100 μl of RPMI-1640 complete medium supplemented with 10% pooled human AB serum and added to the target cells. The plates were then incubated at 37° C in 5% CO_2 for 4 or 16 h. Supernatant was harvested for gamma counting with the use of harvester frames (Skatron, Inc., Sterling, VA). Determinations were carried out in triplicate, and standard deviations were calculated. Specific lysis was calculated with the use of the following formula (all values in cpm):

$$\% \text{ lysis} = \frac{\text{Observed release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

Spontaneous release was determined from wells to which 100 μl of RPMI-1640 complete medium was added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

Detection of Cytokines.

Supernatants of T cells exposed for 24 h to peptide-pulsed autologous EBV-transformed B cells, in IL-2-free medium at various peptide concentrations, were screened for secretion of IFN-γ using an ELISA kit (R & D Systems, Minneapolis, MN). The results were expressed in pg/ml. A CBA system (BD PharMingen, San Diego, CA) was also used to determine the secretion of multiple cytokines by specific T cells. The CBA system uses the fluorescence detection by flow cytometry to measure soluble analytes in a particles-based immunoassay. The BD human Th1/Th2 cytokine

CBA Kit was used to measure IL-2, IL-4, IL-5, IL-10, TNF-a protein levels in a single sample. The cytokine capture beads were mixed with PE-conjugated detection antibodies and then incubated with recombinant cytokine standards or test samples to form sandwich complexes. The sample results were generated in graphic and tabular format, using BD PharMingen CBA analysis software. The results were expressed in pg/ml.

Statistical Analysis.

Statistical analysis of differences between means was done using a two-tailed paired *t* test (Stat View statistical software, Abacus Concepts, Berkeley, CA).

Toxicity Grading - The NCI Common Toxicity Scale.

* If Grade I- II toxicities occur, the patient may continue with the infusion schedule.

* If Grade III toxicity occurs, the "drug" is held until the toxicity decreases to Grade I or II, then the infusion is restarted. If Grade III or IV toxicity occurs after the restart, the "drug" infusions are stopped.

* If Grade IV toxicity occurs, the patient is scored as having Grade IV toxicity and the next infusion is reduced to the previous dose. If the previous dose causes Grade IV toxicity, then the "drug" is stopped.

* If Grade IV toxicity occurs in 1 of 3 patients at a specific dose level, an additional 3 patients are entered at that cell-dose level for a total of 6 patients at that dose level. If 2 of 6 patients at a cell-dose level develop Grade IV toxicity, this dose is defined as the MTD. The next 3 patients are given 66% (two-thirds) of the previous cell-dose level. For the purposes of evaluation for dose-escalation, each patient at the same dose level must have received at least 4 of 6 infusions.

Example 1: Novel MUC-1 Binding Motifs

The primary amino acid sequence of human MUC-1 was analyzed for consensus motifs for novel HLA-A2 binding peptides. Twelve 10-mer peptides were identified, consequently synthesized, and studied for binding to the HLA-A2 molecule in a T2 cell binding assay. The amino acid sequences and positions of these 10-mer peptides are

shown in Table 1. The CEA CAP1-6D peptide and a NCA peptide were used as a positive and negative control, respectively. The predicted binding of the 12 peptides are also given in Table 1. Three of these peptides (P-92, P-94 and P-1108) were shown to have the highest level of binding in the T2 assay.

5

Table 1. Binding of human MUC-1 peptides to HLA-A2 molecules

Peptide	Amino acid Position in MUC-1	Sequence	Predicted binding to HLA-A2*	T2 binding[#]
P-92	92-101	ATWGQDVTSV	POS	740
P-94	94-103	WGQDVTSVPV	NEG	591
P-1108	1108-1117	REGTINVHDV	NEG	482
P-4	4-13	GTQSPFFLLL	NEG	467
P-1105	1105-1114	LAFREGTINV	NEG	461
P-1004	1004-1013	TLASHSTKTD	NEG	442
P-1069	1069-1078	LQRDISEMFL	NEG	433
P-1162	1162-1171	ALLVLVCVLV	POS	431
P-1135	1135-1144	TISDVSVSDV	POS	422
P-1172	1172-1181	ALAIVYLIAL	POS	372
P-1169	1169-1178	VLVALAIVYL	POS	369
P-1177	1177-1186	YLIALAVCQC	POS	338
CAP1-6D	NA	YLSGADLNL	POS	975
NCA	NA	YRPGENLNL	NEG	365

* Predicted binding on the basis of reported motif (37); POS = positive; NEG = negative.

Results are expressed in relative fluorescence.

CAP1-6D is an HLA-A2 binding carcinoembryonic antigen peptide that was used as a positive control.

NCA peptide was used as a negative control.

NA= not applicable.

5

Example 2: Establishment of MUC-1 Specific T cell Lines

Studies were then conducted to determine if MUC-1–specific T-cell lines could be established from PBMCs of an apparently healthy donor. To accomplish this,

10   autologous DCs infected with rF-MUC-1/TRICOM were used as APC. rF-MUC-1/TRICOM is a replication-defective avipox vector containing the transgenes for MUC-1 and for a triad of human costimulatory molecules (B7-1, ICAM-1 and LFA-3, designated TRICOM). rF-MUC-1/TRICOM was shown to efficiently infect human DCs and hyperexpress each of the costimulatory molecules, as well as MUC-1, on the DC

15   surface (Table 2). Approximately 96% of the cells were CD11c and MHC-class II positive.

Table 2.   Phenotypic analysis of DCs infected with rF-MUC-1/TRICOM

Dendritic cells infected with	CD80	CD54	CD58	Class I	MUC-1
Uninfected	4.8 (13.6)	59.5 (62.3)	68.1 (18.2)	99.7 (271.8)	5.0 (95.7)
FP/WT	7.4 (13.3)	79.3 (83.4)	74.0 (19.5)	99.5 (176.3)	3.1 (50.7)
rF-MUC-1/TRICOM	30.9 (35.7)	84.5 (133.9)	79.8 (27.4)	99.9 (189.3)	31.6 (213.1)

20   Flow cytometric analysis of surface marker expression on DCs. DCs used were cultured in AIM-V medium containing 100 ng/ml of rhGM-CSF and 20 ng/ml of rhIL-4 for 7 days. DCs used for infection with FP/WT or rF-MUC-1/TRICOM were cultured as described in "Materials and Methods." Results indicate the percentage of positive cells; numbers in parentheses represent MFI.

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The specificity of the MUC-1–specific T cells generated (designated T-1191-MUC-1) was analyzed after IVS cycle 3 (see Materials and Methods) for their ability to release IFN-γ after stimulation with autologous B cells pulsed with each of the MUC-1 peptides listed in Table 1. The results shown in Table 3 demonstrate that when the T-1191-MUC-1 cells were stimulated with autologous B cells pulsed with peptides P-92, P-1135, P-94, P-1004, P-1069 and P-4, the T cells produced IFN-γ, while the use of autologous B cells pulsed with the other peptides did not result in IFN-γ production. The results shown in Tables 1 and 3 demonstrate that the P-92 peptide had the highest level of T2 binding, as well as the ability to activate T-1191-MUC-1 cells to produce the highest levels of IFN-γ; this peptide was thus chosen for further study.

Table 3.   Production of IFN–γ by T-1191-MUC-1 cells stimulated with autologous B cells pulsed with MUC-1 peptides

| Peptide | Production of IFN-γ (pg/ml) | |
	With peptide	No peptide
P-92	380.8	<52.3
P-1135	347.2	<52.3
P-94	323.6	<52.3
P-1004	305.6	<52.3
P-1069	288.0	<52.3
P-4	260.0	<52.3
P-1105	<52.3	<52.3
P-1108	<52.3	<52.3
P-1162	<52.3	<52.3
P-1169	<52.3	<52.3
P-1172	<52.3	<52.3
P-1177	<52.3	<52.3

5   T-1191-MUC-1 cells were used as effectors at IVS-3. T cells were stimulated with irradiated autologous EBV-transformed B cells pulsed with different MUC-1 peptides at a concentration of 25 µg/ml, and an effector-to-APC ratio of 1:3. Twenty-four-hour culture supernatants were collected and screened for the secretion of IFN-γ.

Example 4: Peptide Analysis

Analysis of the primary and secondary HLA-A2 anchor amino acid residues at positions 2 and 10 of the P-92 peptide revealed that modification of amino acids at these positions could potentially enhance the binding ability of the peptide to the HLA-A2 molecule. Thus, six different analogues of P-92 were synthesized, as shown in Table 4, and tested for their binding ability to T2 cells along with the native P-92 peptide. The CEA CAP-7 (HLA-A3 binding peptide), which has previously been shown not to bind to HLA-A2, was used as a negative control. As shown in Table 4, four of the six analog peptides bound to HLA-A2 at higher levels than the P-92 peptide. Analogues P-93L and P-93I bound HLA-A2 with greatest efficiency.

Table 4.   MUC-1 peptide analogues

Amino acid sequence	Initial designation	T2 binding*
ATWGQDVTSV	P-92 (native)	510
A*I*WGQDVTSV	I-93	823
A*L*WGQDVTSV	L-93	821
A*L*WGQDVTS*L*	L-93/L-101	736
A*M*WGQDVTSV	M-93	723
A*M*WGQDVTS*L*	M-93/L-101	325
A*I*WGQDVTS*L*	I-93/L-101	280

5   Amino acid sequences of the parental P-92 peptide (amino acid positions 92-101 of MUC-1) and analogue
peptides. Amino acids are shown by the single-letter code. Substitution amino acids are indicated in bold
and italic.
*Results are expressed in relative fluorescence values. HLA-A3 peptide (T2 binding=200) was used as a
negative control and CAP1-6D (T2 binding =875) was used as a positive control.

10

Experiments were then conducted to compare the ability of the P-93L and P-93I peptides to bind HLA-A2 at various peptide concentrations. As seen in Figure 1, the P-93L and P-93I peptides bound to HLA-A2 at higher levels than P-92 at all peptide concentrations. The levels of binding were similar for P-93L and P-93I at the various

5   peptide concentrations. These data thus indicated that both P-93L and P-93I with modification in the primary anchor position 2 (position 93 of the MUC-1 molecule) were potential agonists of peptide P-92.

Example 5: Stability of Peptide-MHC Complex.

10   The stability of the peptide-MHC complex for peptides P-92 (native), P-93L and P-93I, was examined. Peptides were incubated with T2 cells overnight, the unbound peptides were washed off, and then incubated with brefeldin-A to block delivery of new class I molecules to the cell surface; at various time points cells were analyzed for the presence of peptide-HLA-A2 complexes. As shown in Figure 2, P-93L-HLA-A2 and P-

15   93I-HLA-A2 complexes were more stable than P-92-HLA-A2 complexes over the 8-hour observation period, with P-93L-HLA-A2 complexes slightly more stable than the P-93I-HLA-A2 complexes over the same period of time. Thus, both the binding of peptides to MHC and the stability of the peptide-MHC complex were shown to be greater for the P-93L and P-93I peptides than the native P-92 peptide.

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The ability of the P-93L and P-93I agonist peptides at various peptide concentrations, to activate the T-1191-MUC-1 cells, was compared. As seen in Figure 3, at each concentration of peptide, pulsing of APC with the P-93L peptide led to the greatest level of IFN-γ production by T-1191-MUC-1 cells as compared with the P-93I

25   peptide or the native P-92 peptide. The P-93L agonist peptide was thus chosen for further study. The cytokine profile of T-1191-MUC-1 cells stimulated with APCs pulsed with either the P-92 or the P-93L peptide was then analyzed. A CBA assay was used for the analysis. Table 5 shows the levels of each of the six cytokines produced by T-1191-MUC-1 cell line stimulated with APCs pulsed with no peptide, P-92 peptide and

30   P-93L peptide. These results demonstrated greater production of type 1 cytokine IL-2 and IFN-g by T cells stimulated with the P-93L peptide than with the P-92 peptide. Low

or undetectable levels of type 2 cytokines IL-4 and IL-10 were seen with either peptide. No TNF-a could be detected in the supernatants at the 24-h time point.

Table 5   CBA assay for the production of cytokines by MUC-1 peptide-stimulated T cells

Peptide	Cytokines.					
	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
None	<20	<20	<20	<20	<20	<20
P-92	58.8	<20	<20	<20	<20	266
P-93L	366.9	<20	140.9	<20	<20	650

The production of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ was analyzed. Standards at concentrations of each cytokine at 0 pg/ml, 312 pg/ml and 5000 pg/ml were used to determine the concentrations of these six cytokines in the samples. T-1191 MUC-1 cells at IVS-3 were used as effectors. T cells were stimulated with irradiated autologous EBV-transformed B cells pulsed without peptide or with P-92 or P-93L peptides at a concentration of 25 μg/ml, and an effector-to-target ratio of 1:3. Twenty-four-hour culture supernatants were collected and screened for the secretion of cytokines. Results are expressed in pg/ml/10^6 cell/ml.

To further compare the biologic activity of the native P-92 peptide and the agonist P-93L peptide, two additional T-cell lines were established. This was accomplished using as APC autologous DCs infected with rF-MUC-1/TRICOM and autologous PBMCs as effectors from an apparently healthy donor. After three IVS, the T-cell lines were stimulated with autologous B cells pulsed with either the P-92 or the P-93L peptide. These two cell lines were designated T-1191-P-92 and T-1191-P-93L, respectively. The two cell lines were shown to be >98% CD8 positive, 99% CD49d positive, <2% CD56 positive and > 75% CD45RO positive cells. The two T-cell lines were then analyzed for their ability to lyse peptide-pulsed targets. T-1191-P-93L was shown to lyse C1R-A2 cells pulsed with P-93L peptide to a greater extent than cells pulsed with the P-92 peptide (Figure 4, squares). T-1191-P-92 also lysed target cells pulsed with the P-93L peptide to a greater extent than those pulsed with the P-92 peptide (Figure 4, triangles). The data in Figure 4 also show that when target cells are pulsed with the native peptide, the T-cell line established with the agonist P-93L peptide lyses target cells at greater levels than the T-cell line established with the native peptide. This

was seen at two different E:T ratios. T-1191-P-93L cells and T-1191-P-92 cells did not lyse C1R-A2 cells when pulsed with control CEA CAP1-6D peptide (Figure 4, circles).

Example 5: Target Cell Recognition.

5   Studies were the conducted to determine whether these two T-cell lines could lyse the MUC-1 positive and HLA-A2 positive breast carcinoma cell line MCF-7. The MUC-1 negative HLA-A2 positive SK-Mel-24 melanoma cell line was used as a negative control. As shown in Table 5, MCF-7 cells were lysed by both the T-1191-P-92 and T-1191-P-93L cells. No lysis was observed against the SK-Mel-24 cells. T-

10   1191-P-93L cells were shown to lyse MCF-7 cells to a greater degree as compared with the T-1191-P-92 cell line. The addition of unlabeled C1R-A2 cells pulsed with the corresponding MUC-1 peptide, but not the CEA CAP1-6D control peptide, decreased the cytotoxic activity of both T-cell lines, demonstrating the MUC-1 specificity of the lysis (Table 5). The cytotoxic activity of these T-cell lines against MCF-7 cells was also

15   shown to be HLA-A2-restricted, as demonstrated by the inhibition of lysis with the addition of anti-HLA-A2 antibody, but not with the control antibody UPC-10 (Table 6).

Table 6   Ability of MUC-1–specific T-cell lines T-1191-P-92 and T-1191-P-93L to lyse a MUC-1–expressing tumor cell line (MCF-7)

Target	T-1191-P-92	T-1191-P-93L
MCF-7	16.5 (2.7)*	24.5 (4.5)*
MCF-7 + C1R-A2	15.6 (3.2)*	21.6 (2.9)*
MCF-7 + C1R-A2 + P-92	4.2 (2.2)	6.1 (1.9)
MCF-7 + C1R-A2 + P-93L	3.0 (1.5)	3.5 (1.2)
MCF-7 + C1R-A2 + CAP1-6D	17.1 (3.8)*	20.8 (3.9)*
SK-Mel-24	0.5 (1.1)	1.4 (0.8)

Results are expressed in % of specific lysis at E: T = 25:1. The numbers in parentheses are the standard deviation. MCF-7 (human breast carcinoma cell line) cells are MUC-1 positive and HLA-A2 positive. SK-Mel-24 (melanoma) cells are MUC-1 negative and HLA-A2 positive. T-1191-P-92 cells and T-1191-P-93L cells were used at IVS-5. The T-1191-P-92 cell line was passaged on the native P-92 peptide, while

25   the T-1191-P-93 cell line was passaged on the agonist P-93L peptide, from IVS 3 to IVS 5. MCF-7 cells

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were labeled with ^{111}In. Labeled MCF-7 cells and unlabeled C1R-A2 cells were used at a ratio of 1:10. C1R-A2 cells were incubated with or without P-92 peptide (25µg/ml), P-93L peptide (25µg/ml) or CAP1-6D control peptide (25µg/ml).

* Statistically significant lysis ($p<0.01$, two-tailed t test) when comparing lysis of MCF-7 cells versus SK-Mel-24 cells. There is also a statistical significance ($p<0.01$, two-tailed t test) when comparing lysis of MCF-7 + C1R-A2 versus MCF-7 + C1R-A2 + P-92 peptide or MCF-7 + C1R-A2 versus MCF-7 + C1R-A2 + P-93L.

The cytotoxic activity of these T-cell lines against MCF-7 cells was also shown to be HLA-A2–restricted, as demonstrated by the inhibition of lysis with the addition of anti-HLA-A2 antibody, but not with the control antibody UPC-10 (Table 7).

The T-1991-P-92 and T-1191-P-93L T-cell lines were derived from an apparently healthy individual. Studies were then conducted to determine if additional T-cell lines could be established from two patients with pancreatic cancer (patients 23 and 18). Four MUC-1–specific T-cell lines were generated and were designated T-23-P-92, T-23-P-93L, T-18-P-92 and T-18-P-93L. The T-cell lines T-18-P-92 and T-18-P-93L were generated from patient 18 by stimulation of PBMCs with autologous DCs pulsed with the P-92 and P-93L peptides, respectively. T-cell lines T-23-P-92 and T-23-P-93L were generated from patient 23 by stimulation of PBMCs by autologous DCs pulsed with the P-92 and P-93L peptides, respectively. As seen in Table 8, all four T-cell lines from both pancreatic cancer patients could be stimulated to produce IFN-γ when stimulated with DCs pulsed with either the P-92 or the P-93L peptide. No IFN-γ production was observed, however, when these T cells were stimulated in a similar manner with the CEA peptide CAP1-6D. For all four T-cell lines, greater levels of IFN-γ production were seen when the P-93L agonist peptide was used as compared with the P-92 native peptide. It also should be noted that the T-cell lines derived using the agonist peptide always showed higher levels of stimulation than the T-cell lines derived from the native peptide, when a given peptide was used for stimulation.

Table 8   Production of IFN-γ by T-cell lines generated from two pancreatic cancer
           patients, stimulated with P-92, and agonist P-93L peptide

T-cell line	Peptide		
	P-92	P-93L	CAP1-6D
T-23-P-92	299.8	644.5	<26
T-23-P-93L	400.5	973.0	<26
T-18-P-92	168.0	366.6	<26
T-18-P-93L	378.2	524.1	<26

5   ᵃResults are expressed as pg IFN-γ produced.
Cells from four MUC-1–specific T-cell lines established from two pancreatic cancer patients (patients 23
and 18) were used as effector cells at IVS-4. These T-cell lines were established by stimulation with P-92-
pulsed autologous DCs (T-23-P-92 and P-18-P-92) or P-93L pulsed autologous DCs (T-23-P-93L and T-
18-P-93L). For IFN-γ production, T-cell lines were stimulated with irradiated HLA-A-positive allogeneic
10   DCs pulsed with either P-92 or P-93L peptide at a concentration of 25 μg/ml and an effector-to-APC ratio
of 10:1. Twenty-four-hour culture supernatants were collected and screened for the secretion of IFN-γ.

         Example 6: Cytolysis of targets by Cancer Patient Derived Cell Lines.

15   Studies were then conducted to determine if the T-cell lines derived from cancer

patient 23 could lyse MUC-1 positive HLA-A2 positive cancer cells. The SK-Mel-24

cell line (MUC-1 negative and HLA-A2 positive) was used as a negative control for

specificity. As can be seen in Table 9 and 10, the T-23-P-93L line, T-23-P-92 line, T-

18-P-93L line and T-18-P-92 line showed lysis of the MCF-7 carcinoma cells at two

20   different E:T ratios, but showed no lysis of the melanoma cell line. In concordance with

the results shown above, the T-cell line (T-23-P-93L, T-18-P-93L) derived using the

agonist peptide demonstrated greater lysis of the tumor cells than the T-cell line (T-23-P-

92, T-18-P-92) derived using the native peptide. This was seen at two different E:T

ratios.

25

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Table 9   Ability of T-cell lines from a pancreatic cancer patient, generated with
agonist peptide P-93L, to lyse cancer cells expressing native MUC-1

T-cell line	Target	E: T Ratios	
		25:1	12.5:1
T-23-P-93L	MCF-7	24.6 (1.0)*#	17.9 (0.2)*#
T-23-P-93L	SK-Mel-24	1.3 (1.1)	0.8 (0.6)
T-23-P-92	MCF-7	14.4 (0.6)*	10.1 (0.8)*
T-23-P-92	SK-Mel-24	0.5 (1.6)	0.4 (1.9)

*Statistical significance (P<0.01, two-tailed *t* test) when comparing lysis of MCF-7 cells versus SK-Mel-24 cells by T-23-P-93L and T-23P-92 cells.
#Statistical significance (P<0.01, two-tailed *t* test) when comparing lysis of MCF-7 cells by T-23-P-93L and T-23-P-92 cells.
Results are expressed as % lysis.

Table 10   Ability of T cells from a pancreatic cancer patient generated with agonist
peptide P-93L to lyse cancer cells expressing native MUC-1

T-cell line	Target	E: T Ratios	
		25:1	12.5:1
T-18-P-93L	MCF-7	2.5 (0.8)*#	14.6 (0.5)*#
T-18-P-93L	SK-Mel-24	1.1 (0.9)	1.0 (0.8)
T-18-P-92	MCF-7	12.1 (0.3)*	8.4 (0.4)*
T-18-P-92	SK-Mel-24	0.9 (1.4)	1.0 (1.2)

*Statistical significance (P<0.01, two-tailed *t* test) when comparing lysis of MCF-7 cells versus SK-Mel-24 cells by T-18-P-93L and T-18-P-92 cells.
#Statistical significance (P<0.01, two-tailed *t* test) when comparing lysis of MCF-7 cells by T-18-P-93L and T-18-P-92 cells.

All documents mentioned herein are incorporated herein by reference.

The foregoing description is illustrative thereof, and it will be understood that variations and modifications can be effected without departing from the scope or spirit of the invention as set forth in the following claims.

5

What is claimed:

1.   An isolated nucleic acid molecule which encodes an agonist polypeptide antigen derived from MUC-1, wherein the agonist polypeptide stimulates an immune response.

2.   The nucleic acid molecule of claim 1, wherein the agonist polypeptide binds to HLA molecules with a high avidity.

3.   The nucleic acid molecule of claim 1, wherein the agonist polypeptide has a higher association constant (K_a) for the HLA than a native polypeptide.

4.   The nucleic acid molecule of claim 1, wherein the agonist polypeptide has a lower dissociation constant (K_d) for the HLA than a native polypeptide.

5.   The nucleic acid molecule of claim 1, which encodes an agonist polypeptide up to about 12 amino acids in length.

6.   The nucleic acid molecule of claim 1, wherein the agonist polypeptide is derived from a mucin tumor antigen.

7.   The nucleic acid molecule of claim 1, wherein the agonist polypeptide is derived from a non-variable number of tandem repeats region of MUC-1.

8.   The nucleic acid molecule of claim 1, wherein the immune response is a cellular immune response.

9.   The nucleic acid molecule of claim 1, wherein the cellular immune response is a cytotoxic T cell response.

10.   The nucleic acid molecule of claim 1, wherein the cellular immune response is a T helper cell response.

11.   The nucleic acid molecule of claim 1, wherein the cellular immune response is a B cell immune response.

12.   The nucleic acid molecule of claim 1, comprising a nucleic acid sequence corresponding to any one of the amino acid sequences as identified by SEQ ID NO: 1 through 23, fragments or variants thereof.

13.   The nucleic acid molecule of claim 1, comprising a nucleic acid sequence corresponding to the amino acid sequence as identified by SEQ ID NO: 2, or fragments thereof.

14.   An isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1 through 23, fragments or variants thereof.

16.   The isolated polypeptide of claim 14, wherein the polypeptide comprises SEQ ID NO: 2, fragments or variants thereof.

17.   The isolated polypeptide of claim 14, wherein the polypeptide binds to HLA molecules with a high avidity.

18.   The isolated polypeptide of claim 14, wherein the polypeptide has a higher association constant (K_a) for the HLA than a native polypeptide.

19.   The isolated polypeptide of claim 17, wherein the polypeptide has a lower dissociation constant (K_d) for the HLA than a native polypeptide.

20.   The isolated polypeptide of claim 17, wherein the polypeptide is derived from a mucin tumor antigen.

21.   The isolated polypeptide of claim 17, wherein the polypeptide is derived from a non-variable number of tandem repeats region of MUC-1.

22.   The isolated polypeptide of claim 17, wherein the polypeptide induces an immune response.

23.   The isolated polypeptide of claim 17, wherein the immune response is a cellular immune response.

24.   The isolated polypeptide of claim 17, wherein the cellular immune response is a cytotoxic T cell response.

25.   The isolated polypeptide of claim 17, wherein the cellular immune response is a T helper cell response.

26.   The isolated polypeptide of claim 17, wherein the cellular immune response is a B cell immune response.

27.   An agonist polypeptide comprising an amino acid sequence which is at least about 60% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

28.   An agonist polypeptide comprising an amino acid sequence which is at least about 80% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

29.   An agonist polypeptide comprising an amino acid sequence which is at least about 90% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

30.   An agonist polypeptide comprising an amino acid sequence which is up to about 99.9% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

31.   A method for generating an immune response to a MUC-1 tumor antigen comprising administering an isolated nucleic molecule in a therapeutically effective dose sufficient to generate a cellular immune response, wherein the isolated nucleic acid molecule encodes any one of polypeptides identified by SEQ ID NO: 1 through 23.

32.   The method of claim 31, wherein the isolated nucleic acid molecule encodes a polypeptide is at least about 60% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

33.   The method of claim 31, wherein the isolated nucleic acid molecule encodes a polypeptide is at least about 80% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

34.   The method of claim 31, wherein the isolated nucleic acid molecule encodes a polypeptide is at least about 90% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

35.   The method of claim 31, wherein the isolated nucleic acid molecule encodes a polypeptide is at least about 99.9% identical to the amino acid sequence of SEQ ID NO: 1 through 23.

36.   The method of claim 31, wherein the isolated nucleic acid molecule comprises a vector expressing any one of amino acids identified by SEQ ID NO: 1 through 23.

37.   The method of claim 31, wherein the isolated nucleic acid molecule comprises a vector expresses a polypeptide identified by SEQ ID NO: 2.

38.   The method of claim 37, wherein an immune response is generated against a MUC-1 tumor.

39.   The method of claim 31, wherein the immune response is a cytotoxic T cell response.

40.   A nucleic acid vector comprising one or more nucleic acid sequences encoding polypeptides as identified by any one of SEQ ID NO: 1 through 23, operably linked to an inducible promoter.

41.   The nucleic acid vector of claim 40, wherein the vector is a viral vector.

42.   The nucleic acid vector of claim 40, wherein the vector is a plasmid.

43.   The nucleic acid vector of claim 40, wherein the inducible promoter is tissue specific.

44.   A recombinant vector comprising a nucleic acid sequence encoding any one of the polypeptides identified by SEQ ID NO: 1 through 23.

45.   A host cell comprising a vector of any one of claims 40 through 44.

46.   A method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising administering to a patient any one of the peptides identified by SEQ ID NO: 1 through 23.

47.   The method of claim 46, wherein the patient is treated by administrating a peptide which is at least about 60% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

48.   The method of claim 46, wherein the patient is treated by administrating a peptide which is at least about 80% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

49. The method of claim 46, wherein the patient is treated by administrating a peptide which is at least about 90% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

50. The method of claim 46, wherein the patient is treated by administrating a peptide which is at least about 99.9% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

51. A method for treating a patient suffering from or susceptible to a MUC-1 tumor comprising:

   isolating dendritic cells from a patient suffering from cancer; and,

   treating the dendritic cells with one or more of the polypeptides identified by SEQ ID NO: 1 through 23; and,

   administering the treated dendritic cells to the patient.

52. The method of claim 51, wherein dendritic cells are treated with one or more polypeptides which are at least about 60% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

53. The method of claim 51, wherein dendritic cells are treated with one or more polypeptides which are at least about 80% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

54. The method of claim 51, wherein dendritic cells are treated with one or more polypeptides which are at least about 90% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

55. The method of claim 51, wherein dendritic cells are treated with one or more polypeptides which are at least about 99.9% identical to any one of the amino acid sequences identified by SEQ ID NO: 1 through 23.

56.   A method for generating an immune response to a weakly immunogenic antigen comprising administering to an individual a polypeptide with a high avidity for HLA fused to the weak immunogen.

57.   The method of claim 56, wherein the polypeptide comprises the HLA binding fragment of SEQ ID NO: 2.

58.   The method of claim 56, wherein the weak immunogen is a differentiation antigen.

59.   The method of claim 56, wherein the weak immunogen is a tumor antigen.

60.   The method of claim 56, wherein HLA binding fragment of SEQ ID NO: 2 is fused to a carcinoembryonic antigen.

61.   The method of claim 56, wherein the HLA binding fragment of SEQ ID NO: 2 is fused to a viral antigen.

62.   The method of claim 56, wherein the HLA binding fragment of SEQ ID NO: 2 is fused to a self-antigen.

ABSTRACT

Novel MUC-1 epitopes outside the VNTR region are identified. In addition, the first agonist epitope of MUC-1 is described. The employment of agonist epitopes in peptide, protein and vector-based vaccine may well aid in the development of effective vaccines for a range of human cancers.
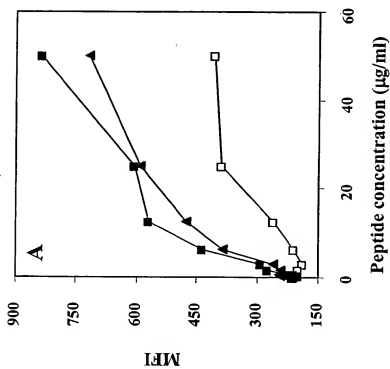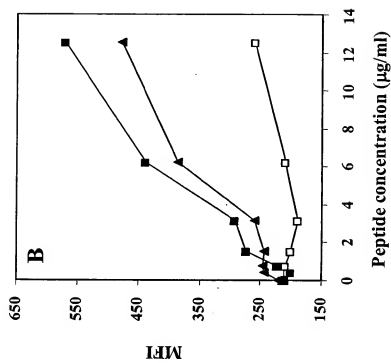
FIGURE 1A

FIGURE 1B

FIGURE 2

FIGURE 3

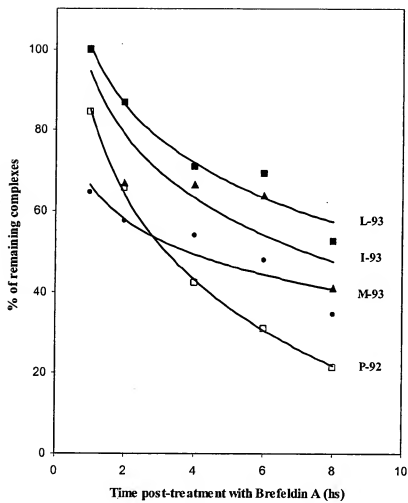
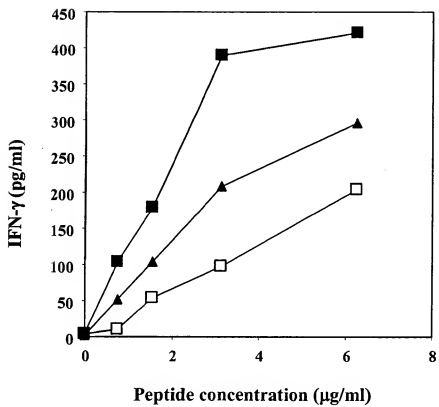Peptide concentration (µg/ml)

FIGURE 4